FEDERAL PATENT COURT

Instructions about a person's right to appeal

An appeal can be filed to the Federal Court of Justice against decisions of the Nullity Senates of the Federal Patent Court. The appeal has to be filed in writing with the Federal Patent Court within

one month

after notification of the decision (Sec. 110, Subsec. 1 PatG [German Patent Act]).

Within the term for filing an appeal an official fee of DM 600,– is to be paid to the cashier of the German Patent Office, indicating the case number and the reason for the payment (appeal fee). If the fee is not paid or not paid in time, the appeal is considered as not having been filed (Sec. 110, Subsec. 1, Sentence 3 PatG).

The appeal fee can be transferred to an account of the cashier of the German Patent Office (*Landeszentralbank München* Account No. 700 010 54 - bank sorting code 700 000 00) or may be paid by means of fee stamps obtainable from the cashier of the German Patent Office. With respect to further modes of payment and the corresponding due dates reference is made to the regulation concerning the payment of fees of the German Patent Office and the Federal Patent Court.

Before the Federal Patent Court of Justice the parties have to be represented by an attorney at law or a patent attorney as an authorized representative (Sec. 121, Subsec. 1 PatG).

BPatG 257
10/91

This is EXHIBIT FIERS-33
to
the Affidavit of Walter C. Fiers
sworn befor me
this 19th day of November, 2001

Commissioner for Oath or Notary Public

FEDERAL PATENT COURT

IN THE NAME OF THE PEOPLE

DECISION

3 Ni 60/95
(file number)

Pronounced on
March 5, 1998
Bandel
Senior Court Secretary
as Registrar
of the Registry

re patent nullity proceedings

of Schering AG, Müllerstraße 170-178, 13353 Berlin, legally represented by its members of the managing board Guiseppe Vita, Klaus Pohle, Hubertus Erlen, Günter Stock, Ulrich Köstlin, ibid.

Plaintiff,

- attorneys of record: Attorney at Law Dr. A. Pünder and associates, Pacellistraße 14, 80333 Munich,

versus

Biogen Inc., Cambridge, Massachusetts, United States of America, legally represented by Chairman and Chief Executive Officer James L. Vincent, ibid.

Defendant,

- attorneys of record: Patent Attorney Dipl.-Chem. Dr. P. Tauchner and associates, Siebertstraße 4, 81675 Munich,

re German Patent DD 160 280

the 3rd Senate (nullity senate) of the Federal Patent Court following the hearing on March 4, 1998 attended by Presiding Judge Grüttemann and judges Dipl.-Chem. Dr. Holzner, Dipl.-Chem. Dr. Wagner, Sredl and Dipl. Ing. agr. Huber

held:

I. Patent DD-AP 160 280, while rejecting the complaint extending beyond that, is partially declared null and void by giving it the following wording:

"1. A method for producing a polypeptide displaying the immunological and biological activity of human fibroblast interferon, characterized by the steps of

(1) culturing an E. coli host transformed by a recombinant DNA molecule, wherein said recombinant DNA molecule is characterized by a DNA insert

(i) selected from the group comprising:

(a) the DNA inserts of G-pPLa-HFIF-67-12[1] (DSM [1]851, DSM 1852), G-pPLa-HFIF-67-12Δ19 (DSM 1853), G-pPLc-HFIF-67-8 (DSM 1854),

(b) DNA sequences which hybridize to any of the foregoing DNA inserts, and

(c) DNA sequences comprising sequences of codons different from those of (a) or (b) but which, on expression, code for a polypeptide having an amino acid sequence identical to that of a polypeptide obtained by expression of codons of any one of the foregoing DNA inserts or sequences,

(ii) said DNA sequence being operatively linked to the major operator and promoter regions of phage λ as expression control sequence, wherein the direction of transcription is co-linear to that of the promoter and nucleotide sequences of the

[1] Translator's note: Here, the spelling used in our correspondence is used. The decision uses different spellings for the DNA inserts.

ß-lactamase or of the MS 2 replicase are located between the promoter and the inserted HFIF sequences; and

(2) collecting said polypeptide.

2. The method according to claim 1, characterized in that the recombinant DNA molecule is selected from the group comprising G-pPLa-HFIF-67-12 (DSM 1851, DSM 1852), G-pPLa-HFIF-67-12Δ19 (DSM 1853), and G-pPLc-HFIF-67-8 (DSM 1854).

3. The method according to claim 1, characterized in that the DNA insert is selected from the group comprising DNA sequences of the formula

ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTTCTCCACTACAGCTC
TTTCCATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGGAGCAATTTTCAGTGTCA
GAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGGACAGGATGAAC
TTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCA
TTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCTA
GCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCATCAGAT
AAACCATCTGAAGACAGTCCTGGAAGAAAACTGGAGAAGAAGATTTCACCAGGGGAA
AACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCATTACCTGAA
GGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAATCCTTAAGGAAC
TTTTACTTCATTAACAGACTTAGAGGTTACCTCCGAAAC, and ATGAGCTACAACTTGCT
TGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCTCCTGTGCAATTGAAT
GGGAGGCTTGAATACTGCCTCAAGGACAGGATGAACTTTGACATCCCTGAGGAGATTA
AGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCATTGACCATCTATGAGATGCTCCA
GAACATCTTTGCTATTTTCAGACAAGATTCATCTAGCACTGGCTGGAATGAGACTATT
GTTGAGAACCTCCTGGCTAATGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGG
AAGAAAAACTGGAGAAGAAGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCT
GAAAAGATATTATGGGAGGATTCTGCATTACCTGAAGGCCAAGGAGTACAGTCACTGT
GCCTGGACCATAGTCAGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTA
CAGGTTACCTCCGAAAC

4. The method of any one of claims 1 to 3, characterized in that the polypeptide is IFN-β.

5. The method according to any one of claims 1 to 4, characterized in that the polypeptide produced is selected from the group comprising polypeptides of the formula:

MetThrAsnLysCysLeuLeuGlnIleAla LeuLeuLeuCysPheSerThrThrAla
LeuSerMetSerTyrAsnLeuLeuGlyPheLeuGlnArgSerSerAsnPheGlnCys
GlnLysLeuLeuTrpGlnLeuAsnGlyArgLeuGluTyrCysLeuLysAspArgMet
AsnPheAspIleProGluGluIleLysGlnLeuGlnGlnPheGlnLysGluAspAlaAlaLeuThrIleTyr
GluMetLeuGlnAsnIlePheAlaIlePheArgGlnAspSerSerSerThrGlyTrp
AsnGluThrIleValGluAsnLeuLeuAlaAsnValTyrHisGlnIleAsnHisLeu
LysThrValLeuGluGluLysLeuGluLysGluAspPheThrArgGlyLysLeuMet
SerSerLeuHisLeuLysArgTyrTyrGlyArgIleLeuHisTyrLeuLysAlaLys
GluTyrSerHisCysAlaTrpThrIleValArgValGluIleLeuArgAsnPheTyr
PheIleAsnArgLeuThrGlyTyrLeuArgAsn, and MetSerTyrAsnLeuLeuGly
PheLeuGlnArgSerAsnPheGlnCasGlnLysLeuLeuTrpGlnLeuAsnGly
ArgLeuGluTyrCysLeuLysAspArgMetAsnPheAspIleProGluGluIleLys
GlnLeuGlnGlnPheGlnLysGluAspAlaAlaLeuThrIleTyrGluMetLeuGln
AsnIlePheAlaIlePheArgGlnAspSerSerSerThrGlyTrpAsnGluThrIle
ValGluAsnLeuLeuAlaAsnValTyrHisGlnIleAsnHisLeuLysThrValLeu
GluGluLysLeuGluLysGluAspPheThrArgGlyLysLeuMetSerSerLeuHis
LeuLysArgTyrTyrGlyArgIleLeuHisTyrLeuLysAlaLysGluTyrSerHis
CysAlaTrpThrIleValArgValGluIleLeuArgAsnPheTyrPheIleAsnArg
LeuThrGlyTyrLeuArgAsn

II. The costs of the law suit are recoverable by ¼ from Plaintiff and by ¾ from Defendant.

III. The decision is preliminarily enforceable with respect to the costs for Plaintiff against a security in the amount of DM 225,000.00 and for Defendant against a security in the amount of DM 75,000.00.

Facts:

Defendant is proprietor of exclusive patent 160 280 (contested patent) filed with the former Amt für Erfindungs- und Patentwesen [AfEP; patent office of the former GDR] of the GDR on April 2, 1981 claiming priorities of earlier applications in Great Britain of April 3, 1980 and June 6, 1980. The application relates to a method for producing a polypeptide having an immunological or biological activity of human fibroblast interferon and comprises nine patent claims. Claim 1 reads in the granted version:

> "1) A method for producing a polypeptide displaying an immunological or biological activity of human fibroblast interferon, characterized in that a host is transformed with a recombinant DNA molecule and cultivated, the recombinant DNA molecule being characterized by a DNA insert selected from the group comprising the DNA inserts of G-pPla-HFIF-67-12, G-pPla-HFIF-67-12Δ19, G-pPlc-H[F]IF-67-8, DNA sequences that hybridize to any of the foregoing DNA inserts, DNA sequences, from whatever source obtained, including natural, synthetic or semi-synthetic sources, related by mutation, including single or multiple, base substitutions, deletions, insertions and inversions, to any of the foregoing DNA sequences or inserts, and DNA sequences which comprise sequences of codons which upon expression code for a polypeptide having an immunological or biological activity similar to that of a polypeptide coded for upon expression of the codons of any of the foregoing DNA sequences and inserts and being operatively linked to an expression control sequence, and collecting the polypeptide."

With regard to the wording of claims 2 to 9 reference is made to the contested patent specification.

Plaintiff, while invoking the provisions of the GDR Patent Act, asserts that Defendant incorrectly claimed the priorities of the earlier applications in Great Britain of April 3, 1980 and June 6, 1980, since the majority of the microorganisms with their plasmids mentioned in the contested patent specification had not been deposited before the application dated. The method according to claim 1 is no longer novel, Plaintiff alleges, pointing in this context to the publications by Taniguchi et al. "Expression of a human fibroblast interferon gene in E. coli", Proc. Natl. Acad. Sci. USA, Vol. 77 (1980), pp. 5230-5233 (Exh. P 10), Derynck et al. "Expression of human fibroblast interferon gene in E. coli", Nature, Vol. 187 (1980), pp. 193 (Exh. P 11), Goeddel et al. "Synthesis of human fibroblast interferon by E. coli", Nucleic Acids Research, 1980, pp. 4057 (Exh. P 12) and Nagata et al. "Synthesis in E. coli of a polypeptide with human leukocyte interferon activity", Nature, Vol. 284 (1980), pp. 316 (Exh. P 13) in conjunction with Kontsek et al. "Antigenic link between human interferons-alpha and -beta: The common Epitope I", Journal of Interferon Research, Vol. 10 (1990), pp. 919 (Exh. P 14).
Plaintiff furthermore holds that the method according to claim 1 is not based on an inventive step. In this regard Plaintiff essentially invokes the publications by Nagata et al. according to Exhibit P 13 and by Taniguchi et al. "The nucleotide sequence of human fibroblast interferon cDNA, Gene 10 (1980), pp. 11-15 (Exh. P 15). The subject matter of claim 1 is suggested at least by a combination of the publications by Taniguchi et al. (Exh. P 15) with Nagata et al. (Exh. P 13) or Goeddel et al. "Direct Expression in Escherichia coli of a DNA sequence coding for human growth hormone", Nature, Vol. 281 (1979), pp. 544 (Exh. P 20) or Roberts et al. "Synthesis of simian virus 40 t antigen in Escherichia coli", Proc. Natl. Acad. Sci. USA 76 (1979), pp. 5596-5600 (Exh. P 23). Also, the method according to the contested patent is not sufficiently disclosed for a person skilled in the art to be worked without undue experimentation.

Plaintiff requests

that patent DD 160 280 be declared null and void.

In the hearing Defendant presented new claims 1 to 5 and explained that it wished to defend the contested patent only in the scope of said claims version. Claim 1 thereof is worded as follows:

"1. A method for producing a polypeptide displaying the immunological and biological activity of human fibroblast interferon, characterized by the steps of

(1) culturing an E. coli host transformed by a recombinant DNA molecule, wherein said recombinant DNA molecule is characterized by a DNA insert

(i) selected from the group comprising:

(a) the DNA inserts of G-pPLa-HFIF-67-12 (DSM 1851, DSM 1852),
G-pPLa-HFIF-67-12Δ19 (DSM 1853),
G-pPLc-HFIF-67-8 (DSM 1854),

(b) DNA sequences which hybridize to any of the foregoing DNA inserts, and

(c) DNA sequences comprising sequences of codons different from those of (a) or (b) but which, on expression, code for a polypeptide having an amino acid sequence identical to that of a polypeptide obtained by expression of codons of any one of the foregoing DNA inserts or sequences,

(ii) said DNA sequence being operatively linked to a lac system, a trp system or the major operator and promoter regions of phage λ as expression control sequence, wherein the direction of transcription is co-linear to that of the promoter; and

(2) collecting said polypeptide."

With respect to the wording of claims 2 to 5 reference is made to the operative part of the decision. Defendant no longer claimed the priority of the British application of April 3, 1980.

Defendant requests

that the complaint be dismissed as far as it is directed to the contested patent in the defended scope.

As an auxiliary measure, Defendant suggests that the claims of the contested patent be worded in accordance with the auxiliary request likewise submitted in the oral hearing the wording of which can be taken from the operative part of the decision. Defendant objects to Plaintiff's argumentation and asserts that the contested patent is patentable in the defended version.

Plaintiff alleges that the method according to the defended claim is inadmissibly broadened since the wording of defended claim 1 represents an aliud vis-à-vis the granted wording with regard to the degeneration of the genetic code. Plaintiff also argued - invoking decision T 0207/94 of the Technical Board of Appeal 3.3.4 of the European Patent Office dated April 8, 1997 in the parallel European patent grant proceedings - that the subject matter of the contested patent in the version now defended was obvious to the person skilled in the art already when relying on her/his expert knowledge.

Grounds for the decision

I.

1) According to § 5(2) Extension Law the 1981 Patent Act is applicable for the exclusive patent granted by the patent office of the former GDR and extended to the entire territory of the Federal Republic of Germany according to § 4 Extension Law. However, as far as the requirements for patentability and duration of protection are concerned, according to the stipulations of Exhibit I Chapter III Special Field E Section II No. 1 § 3(1) Unification Treaty of August 31, 1990 the legal provisions of the former GDR valid at the application date of the contested patent remain applicable for the contested patent (see official reasons re

Extension Law BIPMZ 1992, pp. 213, 223, 224). Hence, as the contested patent was filed before the amendment to the GDR Patent Act of 1983 (BIPMZ 1984, 37), namely on April 2, 1981, the GDR Patent Act of 1950 (BIPMZ 1950, pp. 263) in the version of item 5 of the attachment to the Amendment Act of June 11, 1968 and the Amendment Act of July 31, 1963 (BIPMZ 1963, 275; see also BGHZ 37, pp. 219 - Drahtseilverbindung) is applicable. Accordingly, at the time of filing of the contested patent according to Secs. 1 Subsec. 1, 4 GDR Patent Act of 1950 novelty, technical advance, inventive step and susceptibility to industrial application are essential criteria for the grant of a patent (Otto Kawalle-Günter Schönfeld, Der Patentverletzungsstreit und die Nichtigerklärung von Patenten [patent infringement litigation and the nullification of patents], Schriftenreihe Patentrecht, Vol. 3, Staatsverlag der DDR, Berlin 1967, pp. 110; Adrian et al., Die Anmeldung, Erteilung und Bestätigung von Patenten [The application, grant and confirmation of patents], Schriftenreihe Patentrecht, Vol. 2, Staatsverlag der DDR, Berlin 1967).

2) The admissible complaint already results in the partial nullification of the contested patent in the scope no longer defended by Defendant.

As far as Defendant defends the contested patent in the scope of the main request it could not survive for lack of patentability.

As far as the contested patent is defended in the scope of the auxiliary request the complaint directed thereto had to be dismissed since the Senate could not find that the reasons for nullity of lack of patentability and lack of disclosure set forth by Plaintiff do not present an obstacle to the contested patent under the aspect of lack of enabling working (Secs. 22, Subsec. 1, 21, Subsec. 1, No. 1 and No. 2 Patent Act, §§ 4(1), (5) Extension Act, Secs. 1, Subsec.1, 4 GDR Patent Act of 1950, Secs. 22, Subsec.2, 21, Subsec. 2 Patent Act).

II.

Together with the application of the contested patent with the patent office of the former GDR Defendant claimed the priorities of the British prior applications of April 3, 1980 (BIOGEN I) and of June 6, 1980 (BIOGEN II). Plaintiff holds the view that Defendant incorrectly claimed these priorities and refers in this respect to the requirements for applications of the stipulations of the GDR Patent Act. In

the hearing Defendant declared that it only claims the priority of the application of June 6, 1980 (BIOGEN II). This priority is being correctly claimed. The Board of Appeal 3.3.4 of the European Patent Office, too, arrived at the same conclusion in its decision of April 8, 1997 in the parallel European granting procedure.

The wording of claim 1 according to the main request is already disclosed in somewhat broader form in claims 39, 40 in conjunction with claims 45 and 46 of the priority document "BIOGEN II" referring back to them. In claims 39 and 40 according to "BIOGEN II" the inserts are listed according to feature (a) according to claim 1 (main request). This list is followed by the supplement with regard to the hybridizing DNA sequences according to feature (b). On pages 55 to 60 of the priority document the construction of the three plasmids is described in detail, the inserts of which are defined in claim 1 according to the main claim under (a). On page 81 reference is made to the deposit of the corresponding plasmids in certain E. coli strains with Deutsche Sammlung von Mikroorganismen in Göttingen with the deposit numbers DSM 1851-1854. The deposit was made on June 5, 1980, i.e., one day before the filing date of "BIOGEN II" and hence timely with respect to said patent application so that these plasmids and their inserts and hence a concrete starting material for the hybridization tests were available to the scientific community already on the application date of "BIOGEN II". Feature (c) according to claim 1 of the main request represents a restriction vis-à-vis the DNA sequences mentioned in claim 40 of "BIOGEN II" in the last section (molecules whose DNA inserts comprise codon sequences for a polypeptide with the corresponding properties of the polypeptides coded for by the aforementioned sequences). The use of specific expression control sequences (lac, trp system, major operator and promoter regions of phage λ) according to feature (ii) is described on page 77, last paragraph, to page 78, 1st paragraph of the priority document. The transcription direction of the promoter is unequivocally derivable from the graphic representations (restriction maps) according to Figures 8 to 13 and from the description on page 55, 1st paragraph in "BIOGEN II". The experimental conditions for detecting the biological activity of the target polypeptide are described on pages 63 to 65 and those for detecting the immunological activity on page 72, last paragraph, to page 73 of the priority document.

There are no doubts about the claiming of the priority of June 6, 1980, either, with respect to Sec. 4(e), 4th paragraph of the regulations on the requirements for

drafting and filing invention applications of November 5, 1975 (Exh. P 7). These regulations are not changed by the regulations for the deposit of microorganisms for the filing of invention applications of September 27, 1979 (Exh. P9), since it refers to the deposit of new microorganisms. The contested patent, however, relates to microorganisms which were already known and deposited within the meaning of the above-mentioned Sec. 4 so that the requirements for effectively claiming the priority of June 6, 1980 are met for the feature combination mentioned in claim 1 of the main request.

III.

1) The contested patent relates to DNA sequences, recombinant DNA molecules and a method for producing polypeptides that are similar to human fibroblast interferons, with the DNA sequences being put into practice in corresponding host organisms. Human fibroblast interferon and interferon β are among the so-called class 1 interferons which confer upon cells a resistance to virus infections. Such interferons have been used as antivirus and antitumor agents as well as for immunomodulation and for a short while now for the treatment of multiple sclerosis. Its application as a drug prompted an increase in demand. So far, interferon β has been produced using human cell lines grown in tissue cultures. This method, however, means low yields, bad quality and high costs.

The problem underlying the contested patent is therefore to provide a method for producing interferon β and polypeptides similar to interferon β in high yields, which is cost-effective and in which non-natural sources are used for the production of the interferon β.

Accordingly, claim 1 defended in the main request proposes

> A method for producing a polypeptide displaying the immunological and biological activity of human fibroblast interferon, characterized by the steps of
>
> (1) culturing an E. coli host transformed by a recombinant DNA molecule, wherein said recombinant DNA molecule is characterized by a DNA insert

(i) selected from the group comprising:

(a) the DNA inserts of G-pPLa-HFIF-67-12 (DSM 1851, DSM 1852),
G-pPLa-HFIF-67-12Δ19 (DSM 1853),
G-pPLc-HFIF-67-8 (DSM 1854),

(b) DNA sequences which hybridize to any of the foregoing DNA inserts, and

(c) DNA sequences comprising sequences of codons different from those of (a) or (b) but which, on expression, code for a polypeptide having an amino acid sequence identical to that of a polypeptide obtained by expression of codons of any one of the foregoing DNA inserts or sequences,

(ii) said DNA sequence being operatively linked to a lac system, a trp system or the major operator and promoter regions of phage λ as expression control sequence, wherein the direction of transcription is co-linear to that of the promoter; and

(2) collecting said polypeptide.

2) The voluntary restriction performed in the above defended claims version is admissible.

The restriction in claim 1 to a specific host, namely an E. coli host, and to the three expression control sequences "lac system, trp system or phage λ" is within the scope of the original disclosure and of the granted patent. In contrast to Plaintiff's view this amendment to the claims does not represent a broadening of the scope of protection.

a) The restriction with respect to the host organisms to be transformed to the host E. coli is disclosed in the original description of the example as well as in granted claim 7.

b) While claim 1 as granted generally claims the use of an expression control sequence, the feature group (ii) now contains an admissible restriction to specific expression systems, namely the lactose operon (lac system) and the tryptophan synthetase system (trp system) (each derived from E. coli) as well as the major operator and promoter regions of phage λ. These three specific expression control sequences are mentioned and described in the contested patent specification on page 104, 2nd paragraph with respect to their suitability for expression control of the polypeptide to be produced; the restrictions are originally disclosed, too.

c) The direction of the transcription, namely "co-linear" with that of the promoter is already evident from the set-up described on page 62, lines 1-4 from the bottom of a helper plasmid (p Pla 2311) required for the construction of two of the preferred expression plasmids which helper plasmid carries the promoter of the λ phage. A pointer to the transcription direction of the promoter in a helper plasmid (G-p Plc 24) for the construction of the third preferred expression plasmid can be found on page 63, 2nd paragraph of the contested patent specification. The precursor plasmid G-p Pla-HFIF-67-1 on which all three preferred expression plasmids are based now exhibits the transcription direction described in claim 1 of the main request (cf. page 67, 2nd para. of the contested patent specification). From the graphic representation (restriction maps) both of the plasmids used for the construction of the expression plasmids and the finished expression plasmids the above-mentioned transcription direction is likewise unequivocally recognizable (cf. Fig. 8 to 13 of the contested patent specification).

d) In lines 16 to 20 granted claim 1 was generally directed to DNA sequences comprising codon sequences of any nature which, however, upon expression result in a polypeptide displaying similar immunological or biological activity as a polypeptide with an immunological or biological activity of human fibroblast interferon. In feature (c) of the feature group (i) of claim 1 according to the main request this claim is restricted by the use of DNA sequences having codons that are different from those of (a) or (b) but which lead to an expression product (polypeptide) the amino acid sequence of which is identical to the amino acid sequences of the expression products according to feature (a) or (b). The expression product according to feature (c) must therefore display a specific chemical structure (amino acid sequence), namely that of the expression products of one of the foregoing DNA sequences according to (a) or (b), and is

no longer - as in granted claim 1 - defined exclusively by its immunological or biological activity. Thus, this wording of feature (c) which merely duly considers the known degeneration of the genetic code represents a restriction of the subject matter and the scope of protection of the granted claim. This restriction lies within the scope of what was disclosed and - in contrast to Plaintiff's view - does not lead to an aliud.

3) Claim 1 according to the main request encompasses a method for the production of a polypeptide which should display a biological or immunological activity of human fibroblast interferon. For this purpose, the bacterial host E. coli is provided (transformed) with a plasmid (recombinant DNA molecule).

According to feature complex (i) the plasmid carries structural information signals, i.e., such DNA sequences the translation of which results in a polypeptide with the above-mentioned properties (immunological or biological activity of human fibroblast interferon) and according to feature complex (ii) information signals controlling the translation process of the structural information signal, i.e., so-called expression control sequences.

The structural information signals described under feature group (i) consist according to feature (a) of the DNA inserts of the three preferred expression plasmids with the designations G-pPLa-HFIF-67-12, G-pPLa-HFIF-67-12Δ19 and G-pPLc-HFIF-67-8. This means that according to feature (a) for the DNA sequence (c-DNA) responsible for the structural information for the expression of the polypeptide one of the three plasmids is used, i.e., not the plasmids mentioned therein as a whole. The structural information (DNA insert) required for coding a corresponding polypeptide is introduced according to feature (1) into a recombinant molecule (plasmid or other vector). While under (a) concrete plasmids which were also deposited are mentioned and thus also the sequences of the DNA inserts inherent to said plasmids are determined as structural information, in features (b) and (c) the scope of possible deviations thereof is described.

In feature (b) those DNA sequences are encompassed that hybridize to one of the foregoing inserts, i.e., to one insert of the three plasmids mentioned under (a). For this purpose, the three inserts according to (a) as such have to be presented as single strands. Any DNA sequence capable of hybridizing to these

given sequences according to the principle of base-pairing is thus comprised by said feature. Among these are first and foremost those DNA sequences that are complementary to the inserts presented, i.e., that represent an exact "counterpart" or an exact "copy" of these insert sequences. However, hybridization still takes place when there are only minor differences between the single strands. A satisfactory hybridization of the two single strands to each other to be examined does not take place if one or more longer sections in the one single strand are not complementary to the other single strand. Thus, feature (b) encompasses only those DNA sequences that exhibit minor differences in few sites vis-à-vis the presented sequences defined in (a).

With the content of feature (c) only the so-called "degeneracy of the genetic code" is taken into account. This means that one and the same amino acid may be coded for by different base triplets. Therefore, the structural DNA sequence according to (c), which is different from (a) or (b), may code for a polypeptide which displays an amino acid sequence that a polypeptide coded for by the DNA sequences according to (a) or (b) would display, too.

According to feature group (ii) the expression control sequences belonging to the teaching of the contested patent are mentioned, namely the E. coli derived sequences of the lactose operon (lac system) or the tryptophan synthetase system (trp system) and also the major operator and promoter regions of the phage λ. These are presented as being at the same level as the respective alternative solution by linking them with an "or". Also, the transcription direction of the promoters and of the sequences to be expressed is indicated ("co-linear").

The reference to the collection of the polypeptide according to feature (2) should be understood such that common methods of extraction, enrichment and purification are included. A final processing of the target substance up to the pharmacologically effective product or active ingredient cannot be taken from this wording.

The teaching of claim 1 essentially is that a recombinant DNA molecule is produced for the transformation of E. coli hosts which contains a DNA sequence for the structural expression of a polypeptide displaying the activity of human fibroblast interferon, with said structural DNA sequence being under the control of

the lac or trp system or of the major operator or promoter regions of phage λ for the purpose of its expression.

IV.

The reason of lack of disclosure asserted by Plaintiff as reason for nullity (Sec. 22, Subsec. 1, Sec. 21 Subsec. 1 No. 2 Patent Act) does not apply.

As far as Plaintiff in this context casts doubt on the practicability of the invention by pointing out that the "microorganisms" mentioned in claim 1 under (1)(i)(b) and (c) should also have been deposited, it is stated that these "microorganisms" are DNA sequences that hybridize to the plasmid inserts mentioned under (1) (cf. (b)) or that are different from the DNA sequences (inserts) according to (a) or (b) but code for a polypeptide having an amino acid sequence that is identical to that of the polypeptide that is obtained upon expression of the codon of one of the foregoing DNA sequences and inserts according to (a) or (b). As is evident from the statements under **II.**, this kind of DNA sequences can be detected on the basis of the material according to (b) which was already deposited or with the help of the biological and immunological tests described in the contested patent specification. Therefore, they do not represent inserts of plasmids in microorganisms that have to be deposited under the regulations mentioned under **II.**, above.

The contested patent specification also mentions the routes for the construction of the plasmids mentioned according to feature (a) in claim 1 (pp. 57 to 73). By the deposit of the plasmids it was also ensured that suitable starting material for the hybridization tests according to feature (b) was available to the scientific community. Since the plasmids according to feature (a), which are described in detail and have been deposited, could be the only basis for performing the hybridization tests, the Senate does not see any reason for doubting that suitable DNA sequences hybridizing to one of the inserted sequences according to (a) can be found. The usefulness of the detected further DNA sequences for the expression of a polypeptide having an immunological or biological activity of human fibroblast interferon can be examined by using corresponding tests such as they are described on pp. 81 to 83 and pp. 95, 96 of the contested patent specification after attempts at expression have been made. Thus, the more

detailed description of the stringency of the hybridization in the claims wording is not relevant since additionally a biological or immunological activity of human fibroblast interferon is required which must be referred to for the selection of suitable hybridizing sequences.

With respect to the expression control sequences (feature (ii)) it should be noted that the suitability of the major operator and promoter regions of phage λ for the expression control for the production of the desired polypeptide was confirmed and described in detail (cf. contested patent specification pp. 61). This experimental set-up as well as the suitability of the phage λ promoter for the expression of the polypeptide as such had not been denied by Plaintiff. While the feasibility of expression with the lac or trp system as promoters is not described in detail in terms of its experimental set-up, however, it is sufficient to assume that these systems work since it is not important as a method using these promoters (lac and trp system) is not based on an inventive step.

V.

However, the method according to claim 1 of the main request turns out to be not patentable under Secs. 1, 4 GDR Patent Act of 1950.

1) The subject matter of claim 1 according to the main request is undoubtedly susceptible to industrial application.

According to Sec. 1 GDR Patent Act of 1950 in the version of the Amendment Act of July 31, 1963 and item 5 of the attachment to the Amendment Act of June 11, 1968 (see I.1., above) an invention must be susceptible to industrial application. This prerequisite for protection encompasses two requirements the technical solution must meet: The latter must be practicable for the person skilled in the art in terms of its disclosure on the basis of the "recipe" given in the claim, if necessary by taking recourse to other sections of the patent specification. Also, it must be susceptible to industrial application.

It has already been pointed out and substantiated above that the teaching of claim 1 according to the main request is enablingly disclosed.

The susceptibility to industrial application of the claimed method is also beyond doubt since the method is directed to the production of a pharmacologically active product, or rather creates the basis for such a product. Plaintiff has not denied this fact.

---

2) The subject matter of claim 1 of the main request also meets the requirement of novelty.
According to Sec. 4 GDR Patent Act of 1950 in the version of the above-mentioned amendments acts an invention is not considered novel if - at the time of the application date - it was already described in public references of the last 100 years, otherwise published by the patent office or publicly used within the domestic territory in such a way that afterwards other experts are capable of using it.

The references cited according to Exhibits P 10 to P 12 by Plaintiff (Taniguchi et al., Proc. Natl. Acad. Sci. USA, Vol. 77 (1980), pp. 5230-5233; Derynck et al., Nature, Vol. 187 (1980), pp. 193; Goeddel et al., Nucleic Acids Research, 1980, pp. 4057) inter alia with regard to the novelty question were published after the priority-establishing application date of "BIOGEN II", i.e., after June 6, 1980 and therefore do not represent prior art relevant for the contested patent. Also the purely immunological work according to Exhibit P 14 (Kontsek et al., J. Interferon Res. 10, 1990, pp. 199-128) is post-published and therefore not relevant for the present case.

The publication by Nagata et al., Nature 284, 1980, pp. 315-320 (Exh. P 13) discloses a method for producing human leukocyte interferon so that the method according to claim 1 of the main request differs from this prior art reference already in the production of the desired target substance or target substance group. Furthermore, expression of the human leukocyte interferon is carried out in plasmid p BR 322 with the inserted foreign DNA under the control of the ß lactamase promoter while the subject matter underlying the patent uses other promoter systems according to feature (ii).

The reference according to Exhibit P 15 (Taniguchi et al., Gene 10, 1980, pp. 11-15) contains the complete DNA sequence coding for human fibroblast interferon but not a method for genetically producing said polypeptide. Only a cloning vector (plasmid Tp IF-319-13) as such is described, but this cloning vector does not

contain expression control sequences according to feature (ii) of claim 1 of the main request.

3) The method according to claim 1 of the main request constitutes an advance in the art.

The prerequisite for protection of an advance in the art was derived by the case law from the concept of invention used in the GDR Patent Act. For such an advance it was considered sufficient that at least one advantageous effect of any quality and quantity occurs vis-à-vis the known solution when using a protected solution (cf. Adrian, loc. cit., pp. 151).

The genetically engineered production of human fibroblast interferon in transformed bacteria renders the production of human cell series grown in tissue culture superfluous which requires substantially more time and/or money. It is considerably easier to maintain and propagate the transformed bacteria in culture than the corresponding fibroblast cultures. Plaintiff has not denied this fact.

4) However, the subject matter of claim 1 according to the main request is not based on an inventive step.

Also the prerequisite for protection of inventive merit can be derived from the term "invention" as used in the Patent Act and which was understood in the case law such that an inventive quality can only be asserted "if the new technical teaching suggested by the inventor was not obvious to the person skilled in the art having average skill and knowledge" (Board of Invalidation of August 6, 1960, Announcements of the AfEP 7/1963, p. 190; cf. also Adrian, loc. cit., pp. 154).

In the reference according to Exhibit P 15 (Taniguchi et al., Gene 10, 1980, pp. 11-15), the prior publication of which Plaintiff does not deny and which had already been cited in the priority-establishing document "BIOGEN II", p. 10, ll. 24 to 26, the nucleotide sequence of the human fibroblast interferon cDNA as well as the (predictable) amino acid sequence of the polypeptide are already indicated (Fig. 2). The cDNA with a length of about 800 base pairs was inserted into the EcoRI site of the E. coli plasmid p BR 322. (p. 12, left col., last para.) and resulted in the hybrid plasmid Tp IF 319 - 13. This hybrid plasmid is suitable for the transformation of an E. coli host. In this case the transformation takes place only

in order to take up and propagate said plasmid in the host cells, since it is a cloning vector. However, the hybrid plasmid according to Taniguchi et al. (P 15) is not suitable for effecting expression of the polypeptide in the E. coli host cell since it lacks the expression control sequences with respect to features (ii) according to claim 1. Features (1), (i), (a) to (c), however, are anticipated by said prior art reference.

The expression of the cDNA known from P 15 and inserted into a vector required only suitable expression control sequences (mainly promoters). No inventive activity was required at the priority date for finding such promoters in order to arrive at the teaching of claim 1 of the main request. Due to its complexity and the resulting need to interlink and perform simultaneously various biochemical and microbiological steps in genetically engineered processes the persons skilled in the art are supposed to be a team of bench biochemists, cell biologists, molecular biologists and microbiologists.

At the date of the contested patent this scientific community was very interested in the expression of interferons, which were then rated as promising antivirus and antitumor agents. For example a glance at the relevant literature published shortly after the filing date of the contested patent shows that there was a close neck to neck race for the expression of human fibroblast interferon. In this situation a research team addressed herein as person skilled in the art will perform tests with those expression control sequences that were already described and available at the priority date of the contested patent since the problem of expression - at least at that time - could not be solved by following theoretical approaches alone. The Senate does not fail to see that these persons skilled in the art will perform first preparatory tests for the expression of human fibroblast interferon following the example of Exhibit P 13 (Nagata et al., Nature 284, March 1980, pp. 316-320), in which the expression of human leukocyte interferon, a polypeptide related with human fibroblast interferon, is described. According to said reference human leukocyte interferon cDNA is inserted into the Pst I site of the E. coli plasmid p BR 322 (p. 316, right col., bottom), namely in the same reading frame as the ß lactamase gene which is present on the plasmid anyway (p. 318, left col., 2nd para.) - this gene is responsible for the ampicillin resistance - and downstream of said resistance gene so that the expression control sequence of the ß lactamase gene has the function of a promoter for the expression of the subsequent human leukocyte interferon cDNA. According to

Defendant's argumentation a combination of the teaching according to said reference (P 13) with the teaching according to reference (P 15), which is considered close prior art by Defendant, too, does not result in the expression of human fibroblast interferon. Defendant tries to substantiate this view by submitting a corresponding test report. It may be left undecided whether this is actually true. At least the team of experts in question will not, as Defendant alleges, hold onto this system after a possible failure with the ß lactamase gene and perform the "error search" only within this system. Even if the causes for a failure will not be immediately and definitely identified, the team of experts will include in this error search the question of the promoters as Defendant itself admits in a diagram with regard to the error search submitted during the hearing (cf. "failure → other promoter"). Therefore, the team will carry out the tests with other expression control sequences for the expression of eukaryotic genes in prokaryotic host organisms which were already known at the filing date of the contested patent and hand been repeatedly described.

The pre-described expression systems include both the promoters of the lac and the trp system as is evident from a list of literature references of the eukaryotic genes expressed at the priority date according to Exhibits P 17 or P 14 of Plaintiff which Defendant does not deny in terms of their content. According to this list, the lac and the trp promoter for the expressions of mammalian genes in E. coli are equally suitable. The list according to P 17 contains the pre-published references P 20 (Goeddel et al., Nature 281, pp. 544-548, 1979) and P 23 (Roberts et al., Proc. Natl. Acad. Sci. USA 76, 11, pp. 5596-5600, 1979). In these two references vector constructs (plasmids) are depicted and described with regard to their construction and effect on the expression of human growth hormone (P 20) and Simian Virus 40 t antigen (P 23) in E. coli. In each case the expression is under the control of the lac operon of E. coli. At any rate, it is obvious that the lac system is even suitable for expressing human proteins such as the growth hormone, or virus proteins. The reference according to Exhibit P 51 (Fraser and Bruce, Proc. Natl. Acad. Sci. USA 75, pp. 5936-5940, 1978) discloses the expression of chicken ovalbumin in E. coli using the lac system. Thus, it was obvious to the team of experts dealing with the problem at the priority date to use such promoter systems also for human fibroblast interferon. No prejudice of the scientific community had to be overcome if only because such promoters had already been successfully used for expressing several proteins, i.e., including human proteins.

According to Defendant's argumentation the promoters (lac, trp system, promoter from phage λ) mentioned under (ii) were equally available, which is evident from the list of the possible promoter systems on page 104, 2nd paragraph of the contested patent specification. Specific problems with the lac or trp system are neither alleged nor asserted in the contested patent specification. Rather, Defendant argues that already one successful expression attempt with one of these promoter systems also clears the way for the alternative embodiment. Whether this is actually true or not may be left undecided. In contrast, Plaintiff admits that while the promoter system of the phage λ was known at the priority date of the contested patent it was known only in connection with the expression of prokaryotic genes.

Defendant did not take up a suggestion by the Senate for a restriction of claim 1 to the promoter system of the phage λ and deletion of the lac and trp system. Therefore, the Senate dispenses with making a corresponding amendment to defended claim 1 (main request) ex officio. According to the Senate's decision of July 2, 1992 (BPatGE 35, 127) it is not justified to assess and maintain the contested patent in a version to be elaborated by the Senate which Patentee expressly rejected (loc. cit., p. 128). With regard to this decision the Senate sees itself basically confirmed by the judgment "Schwenkhebelverschluß" of the Federal Court of Justice (GRUR 1997, 272) in which there is the wording "There are many arguments in favor of the belief that it is only for Patentee to defend the granted claim in a restricted version worded by it if the avoidance of a complete nullification of said claim is desired" (loc. cit., p. 273). What is actually important for the Senate is to not maintain a contested patent in a wording which Patentee does not support - at least in the form of an auxiliary request. For it is only Patentee that is in a position to make the deliberations regarding the technical applicability and the enforceability of the patent on the market as well as the effect on infringement proceedings (cf. in this context BGH GRUR 1989, 103, 104 "Gießkannenverschlußvorrichtung"). The Senate always draws the parties' attention to this practice in the oral hearing. Therefore, claim 1 according to the main request with all the alternative embodiments contained therein cannot result in the maintenance of the contested patent in this scope.

VI.

The complaint could not be successful as far as Defendant defends the contested patent with the auxiliary request submitted during the oral hearing.

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1) The defended version of claim 1 of said auxiliary request differs from claim 1 defended with the main request in that the feature according to item (i)(c) reads:

> "(ii) said DNA sequence being operatively linked to the major operator and promoter regions of phage λ as expression control sequence, wherein the direction of transcription is co-linear to that of the promoter and nucleotide sequences of the ß-lactamase or of the MS 2 replicase are located between the promoter and the inserted HFIF sequences; and ..."

Otherwise, the claims are worded identically.

2) The further restriction performed by way of this claims version is admissible.

With respect to the disclosure reference is made to the description pp. 61 of the contested patent specification - which corresponds in this respect to the original application papers - and with respect to the right to priority it is pointed to "BIOGEN II" where the plasmid constructs are described in detail including the P_L promoter of bacteriophage λ. The location of the nucleotide sequences of the ß lactamase or the MS 2 replicase between the promoter and the HFIF sequences is shown in the graphic representation of the expression plasmids according to Figures 11 to 13 of the contested patent specification or of "BIOGEN II". Support of this fact can be found in the contested patent specification on pages 70 and 71, 1st paragraph each, and page 72, last paragraph to page 73, 1st paragraph, where the polypeptide constructs to be expected with or without fusion protein are pointed out. In "BIOGEN II" this feature is evident from the passages on page 57 to 60. With regard to the admissibility of the claim as well as to the practicability of the teaching of said claim - the latter is not doubted by the Senate, because the claims wording is oriented towards the actually performed and described examples - reference is made to the statements in the main request.

3) Claim 1 according to the auxiliary request is patentable. With respect to the question of novelty, technical advance as well as the susceptibility to industrial application reference is made to the statements regarding the main request.

The method according to claim 1 of the auxiliary request is based on an inventive step.

As is evident from the list of the expressions of eukaryotic genes and their pertaining promoter systems carried out until the priority date of the contested patent, which list was submitted as Exhibit P 17, the promoter system of the phage λ was not used. As Plaintiff itself admits the P_L promoter was known at the priority date of the contested patent as expression control sequence, however, only with regard to the expression of prokaryotic genes. Furthermore, the P_L promoter - unlike in the lac or trp system - is an expression control sequence foreign to E. coli, i.e., not originating from said organism. The use of this promoter from phage λ in connection with the nucleotide sequences of the ß lactamase or MS 2 replicase located between the promoter and the cDNA or eukaryotic origin to be expressed cannot be derived from the prior art cited in the present proceedings. Plaintiff was unable to substantiate any circumstances which could have suggested such a use to the assumed team of experts at the priority date of the contested patent.

Claim 1 according to the auxiliary request and the pertaining claims 2 to 5 referring thereto therefore remain valid.

Decision T 0207/94 of the Technical Board of Appeal 3.3.4 of the European Patent Office of April 8, 1997 which resulted in the revocation of the parallel European patent 0 041 313 does not conflict with this since in the appeal proceedings mentioned the decision related to a claim in which the expression control sequence was not more closely defined and neither the transcription direction nor the location of specific protein genes upstream of the polypeptide cDNA was indicated.

V[II].

The decision on the costs is based on Sec. 84 Subsec. 2 Patent Act in conjunction with Sec. 92 Subsec. 1 Code of Civil Procedure and the percentages correspond to the extent to which the parties win or lose.

The decision on the preliminary enforceability is passed on the basis of Sec. 99 Subsec. 1 Patent Act in conjunction with Sec. 709 Code of Civil Procedure.

Grüttemann Dr. Holzner Dr. Wagner Sredl Dr. Huber

EUKARYOTIC GENES CLONED AND EXPRESSED PRIOR TO JUNE 6, 1980

	Gene	Reference	System
MAMMALIAN GENE: *β*-GALACTOSIDASE EXPRESSION SYSTEM			
1	human somatostatin	Itakura et al. (1977)	*β-gal* promoter
2	chicken ovalbumin	Fraser et al. (1978) Mercereau et al. (1978)	*β-gal* promoter
3	mature human insulin	Goeddel et al. (1979a)	*β-gal* promoter
MAMMALIAN GENE: *β*-LACTAMASE EXPRESSION SYSTEM (Inserted into PstI site of pBR322)			
4	rat preproinsulin	Villa-Komaroff (1978)	*bla* promoter
5	mouse dihydrofolate reductase	Chang et al. (1978)	*bla* promoter
6	rat pregrowth hormone	Seeburg et al. (1978)	*bla* promoter
7	human leukocyte interferon (IFN-α)	Nagata et al. (1980)	*bla* promoter
8	mouse Ig light chain	Amster et al. (1980)	*bla* promoter
MAMMALIAN GENE: *TRP D* EXPRESSION SYSTEM			
9	human pregrowth hormone	Martial et al. (1979)	*trp* promoter
MAMMALIAN GENE: *LAC* PROMOTER			
10	human mature growth hormone	Goeddel et al. (1979b)	*lac* promoter[1]
VIRAL GENE (Genes which are expressed normally by infected eukaryotic hosts)			
11	simian virus 40 t antigen	Roberts et al. (1979)	*lac* promoter[2]
12	human hepatitis B virus	Burrell et al. (1979)	*bla* promoter
13	fowl plague virus	Emtage et al. (1980)	*trp* promoter Insert at pBR322 HindIII
YEAST GENE			
14	Neurospora dehydroquinolate hydrolase	Vapnek et al. (1977)	Insert at pBR322 HindIII/EcoRI
15	Saccharomyces *His* and *Leu*	Ratzkin et al. (1977)	ColEi plasmid
16	Saccharomyces galactokinase	Schell et al. (1979)	Insert at pBR322 BamHI
17	Saccharomyces OMP decarboxylase	Bach et al. (1979)	Insert at pBR322 HindIII

Key: *β-gal* = *β*-galactosidase gene
bla = *β*-lactamase gene
trp = tryptophan operon
lac = lactose operon

[1] System subsequently used by Goeddel et al. (Nucleic Acids Research, Vol. 8, No. 18, pp. 4057-4074 (1980)), to express IFN-*β*.

[2] System subsequently used by Taniguchi et al. (PNAS, 77:5230-5233 (September 1980)), to express IFN-*β*.

This is EXHIBIT FIERS-34
to
the Affidavit of Walter C. Fiers
sworn before me
this 19 th day of November, 2001

Commissioner for Oath or Notary Public

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William E. Stewart II

The Interferon System

Springer-Verlag
Wien New York

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to
the Affidavit of Walter C. Fiers
sworn before me
this 19 th day of November, 2001

Commissioner for Oath or Notary Public

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The Interferon System

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charge heterogeneous forms to apparently homogeneous interferons. However, further studies are needed to determine whether these molecules are in fact carbohydrate-free. Stewart II *et al.* (1978) have found discrepancies in the apparent sizes of these chemically cleaved interferons when measured in SDS-gels and in sieving columns, still suggesting glycoprotein character. Similarly, Lin *et al.* (1978) have found evidence of heterogeneity of these apparently homogeneous interferons by assays on heterologous cells.

Havell and his colleagues (1977) have used another approach to determine carbohydrate contributions to human fibroblast interferons. On the basis of their earlier finding that the glycosylation inhibitors 2-deoxy-D-glucose and D-glucosamine partially inhibited interferon production in human fibroblast cultures (Havell *et al.*, 1975b), they determined the size and charge properties of the residual interferon made under such conditions. The interferon made in the presence of either inhibitor was somewhat less charge-heterogeneous than the native interferon in isoelectric focusing gels, focusing at about pH 7.4, and instead of a single peak at about 20,000 daltons in SDS-gels as seen with native fibroblast interferon, small peaks were found at both 20,000 and 16,000 daltons. This suggests that glycosylation of human fibroblast was partially inhibited in glycosylation inhibitors. However, Wiranowska-Stewart and her associates (1978) were unable to reduce the size of human fibroblast interferon by periodate cleavage and such presumably heavily glycosylated fibroblast interferon gave similar molecular weights in SDS-gels and in sizing columns.

Recently, Stewart II *et al.* (1978) have found that treatment of mouse interferons with periodate will apparently convert the SDS-gel pattern of 38,000 dalton interferon to the 22,000 dalton interferon and a small amount of activity appears at 15,000 dalton (Fig. 15). However, when measured in Sephacryl S-200 sizing columns both preparations elute as broad peaks at about 30,000 daltons, suggesting again that the periodate-treated interferon was still behaving anomalously, as would be expected if it were still a glycoprotein. Taking a lead from the earlier finding of Havell *et al.* (1975b) that D-glucosamine or 2-deoxy-D-glucose inhibited most interferon production in fibroblasts, these investigators attempted to determine the size of any residual interferon possibly made in mouse cells in the presence of such inhibitors. In fact, we found that nearly normal quantities of interferons were produced, but virtually all of the interferon migrated at 15,000 daltons in SDS-gels, instead of the normal sizes of 38,000 and 22,000 daltons for mouse interferon, and these preparations of interferon made in glycosylation-inhibitors were also 15,000 daltons in Sephacryl S-200 sizing columns, suggesting they were carbohydrate-free. The aglycointerferon, or "interferoid" behaves identically to native 38,000 and 22,000 dalton apparent molecular weight forms in terms of hydrophobicity on Affigel 202 columns suggesting, in contrast to the finding of Jeng (1977), that carbohydrate content is not related to hydrophobicity of the interferons.

These data suggest that carbohydrate-free interferons are equally as active as native glycosylated interferons. These results are quite important in terms of pending plans for efforts to obtain a small active-core polypeptide of interferon which can be chemically synthesized. It is tempting to speculate that a common active-core exists even for those interferons which are apparently products of different genes; for, as will be described below (Section VII

US005460811A

United States Patent [19]

Goeddel et al.

[11] Patent Number: 5,460,811

[45] Date of Patent: Oct. 24, 1995

[54] **MATURE HUMAN FIBROBLAST INTERFERON**

[75] Inventors: **David V. Goeddel**; **Roberto Crea**, Burlingame, Calif.

[73] Assignee: **Genentech, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **365,284**

[22] Filed: **Jun. 12, 1989**

Related U.S. Application Data

[60] Continuation of Ser. No. 889,722, Jul. 28, 1986, abandoned, which is a division of Ser. No. 291,892, Aug. 11, 1981, abandoned, which is a continuation-in-part of Ser. No. 190,799, Sep. 25, 1980, abandoned.

[51] **Int. Cl.**[6] **C07K 15/26**; A61K 37/66

[52] **U.S. Cl.** **424/85.6**; 424/85.4; 530/351

[58] **Field of Search** 530/351; 424/85.6, 42485.4

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Ex 30 02

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to
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sworn before me
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Primary Examiner—Garnette D. Draper
Assistant Examiner—Shelly Guest Cermak

[57] **ABSTRACT**

A cDNA library is constructed using mRNA from human fibroblasts induced with poly(I):poly(C). A bacterial clone containing fibroblast interferon cDNA sequences identified by hybridization to a cDNA probe synthesized using deoxyoligonucleotide primers which hybridize to fibroblast interferon mRNA specifically. Expression plasmids are constructed which permit the synthesis in *E. coli* of 8×10^7 units of human fibroblast interferon per liter of culture. The bacterially produced fibroblast interferon is indistinguishable from authentic human fibroblast interferon by several criteria.

6 Claims, 6 Drawing Sheets

Protein	1 2 3 4 Met-Ser-Tyr-Asn-	
mRNA	(5') AUG-UC$^{G}_{A}$$_{U}$$_{C}$-UA$^{U}_{C}$-AA$^{U}_{C}$	(16 combinations)
	(5') AUG-AG$^{U}_{C}$-UA$^{U}_{C}$-AA$^{C}_{U}$	(8 combinations)
Complementary DNA primers	ATT-$^{A}_{G}$TA-$^{T}_{C}$GA-CAT	Pool 1
	ATT-$^{A}_{G}$TA-$^{A}_{G}$GA-CAT	Pool 2
	ATT-$^{A}_{G}$TA-$^{A}_{G}$CT-CAT	Pool 3
	GTT-$^{A}_{G}$TA-$^{T}_{C}$GA-CAT	Pool 4
	GTT-$^{A}_{G}$TA-$^{A}_{G}$GA-CAT	Pool 5
	GTT-$^{A}_{G}$TA-$^{A}_{G}$CT-CAT	Pool 6

FIG. 1

FIG. 2A

FIG. 2B

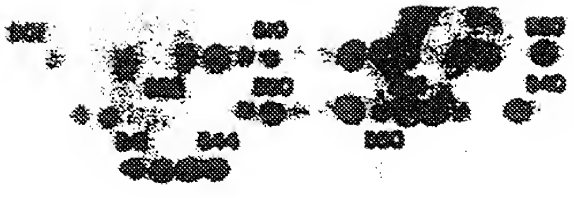

FIG. 2C

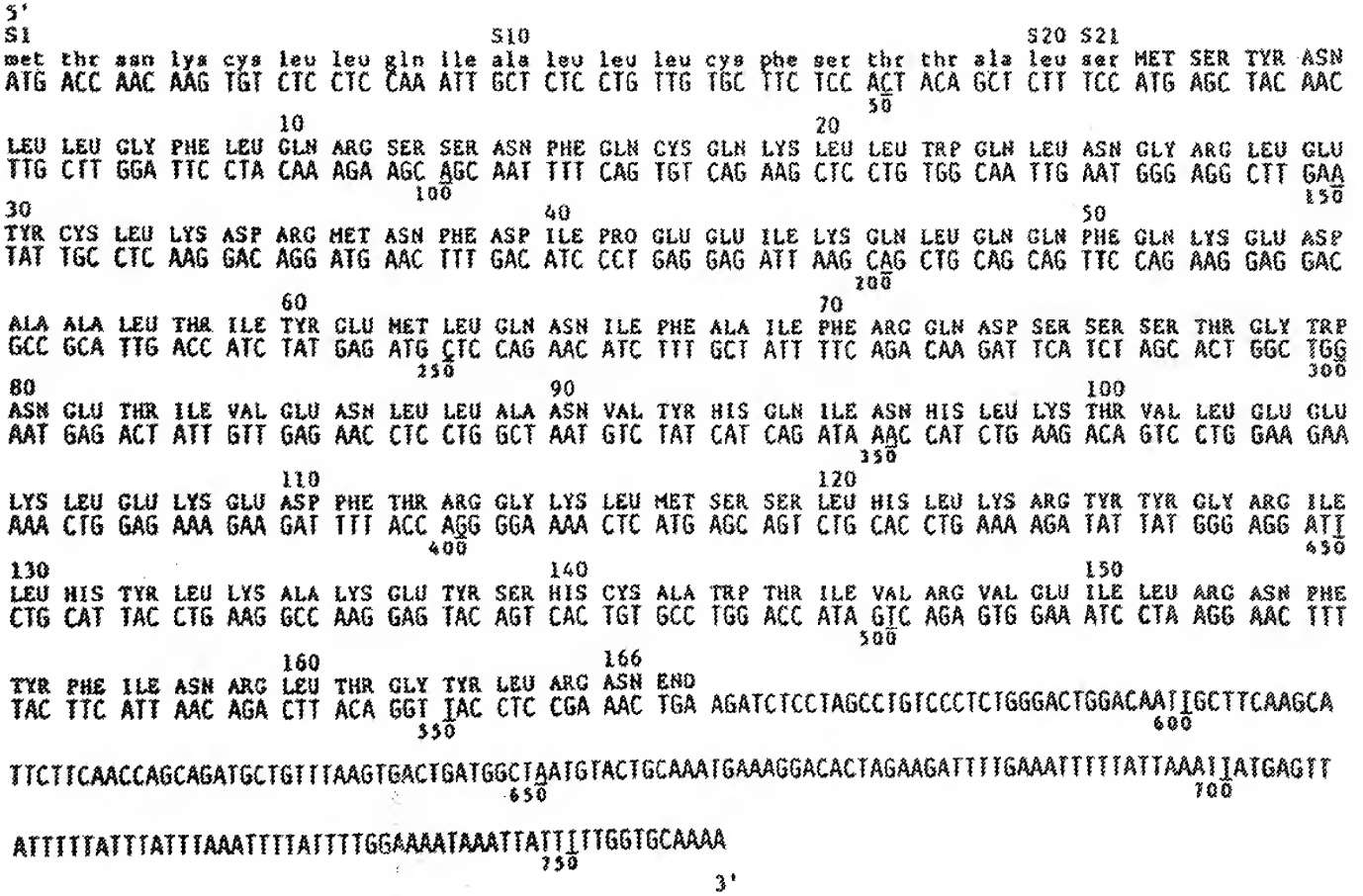

FIG. 3

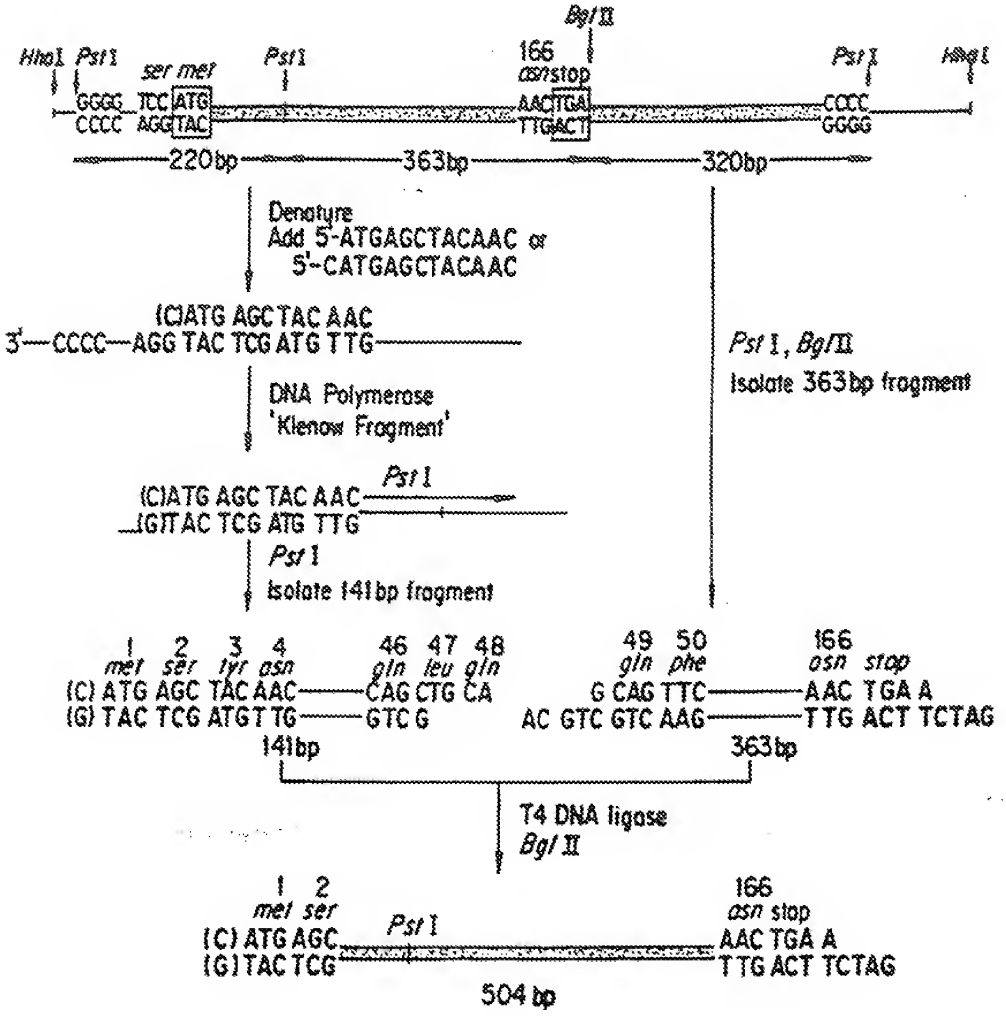
Bgl II
HindI Pst I
ser met
Pst I
166
asn stop
Pst I
HindI
GGGG TCC ATG
CCCC AGG TAC
AAC TGA
TTG ACT
CCCC
GGGG
220bp
363bp
320bp
Denature
Add 5'-ATGAGCTACAAC or
5'-CATGAGCTACAAC
(C)ATG AGC TAC AAC
3'—CCCC—AGG TAC TCG ATG TTG—
Pst I, Bgl II
Isolate 363bp fragment
DNA Polymerase
'Klenow Fragment'
Pst I
(C)ATG AGC TAC AAC
(G)TAC TCG ATG TTG
Pst I
Isolate 141bp fragment
1 2 3 4
met ser tyr asn
46 47 48
gln leu gln
(C) ATG AGC TAC AAC——CAG CTG CA
(G) TAC TCG ATG TTG——GTC G
141bp
49 50
gln phe
166
asn stop
G CAG TTC——AAC TGA A
AC GTC GTC AAG——TTG ACT TCTAG
363bp
T4 DNA ligase
Bgl II
1 2
met ser
166
asn stop
(C) ATG AGC
(G) TAC TCG
Pst I
AAC TGA A
TTG ACT TCTAG
504 bp

FIG.4

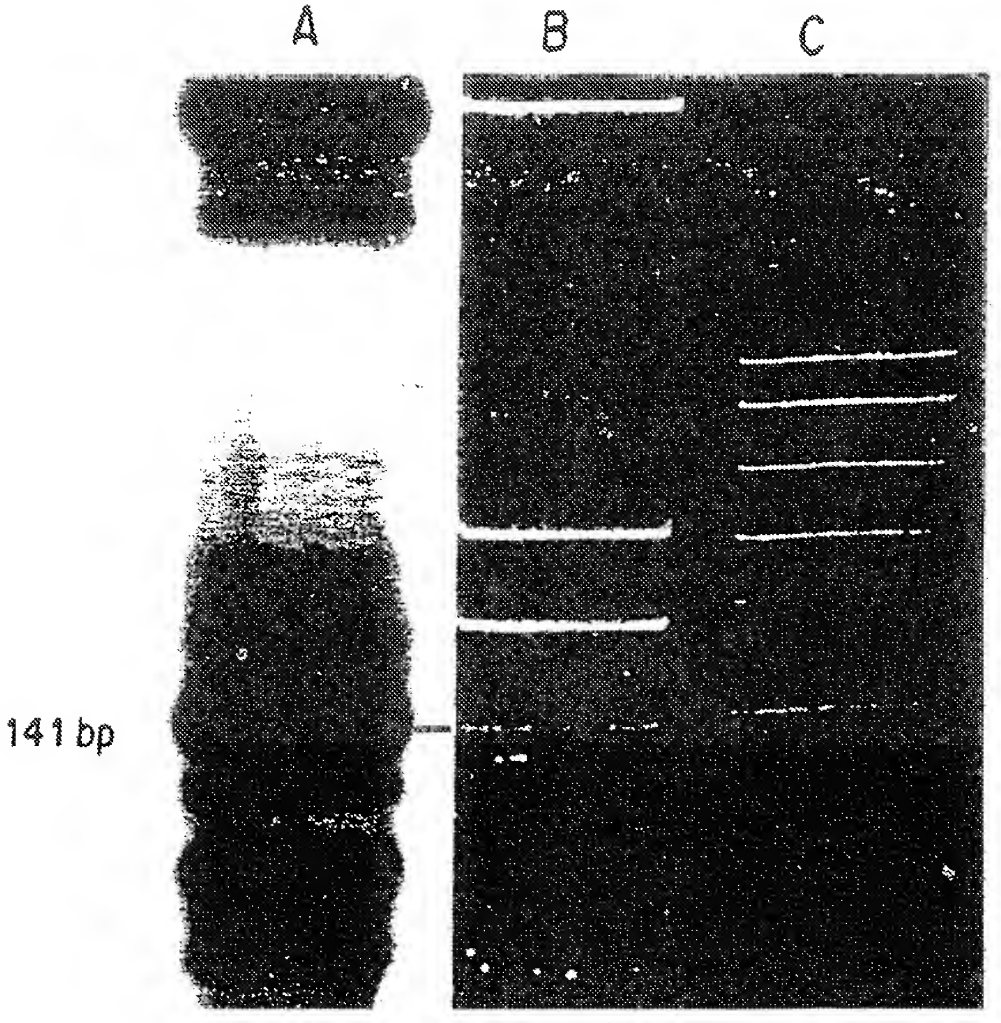

FIG. 5

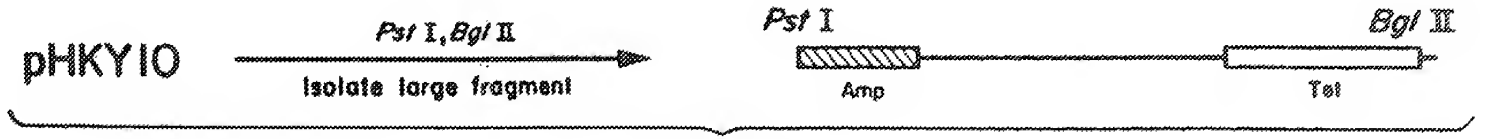

FIG.6(a)

FIG.6(b)

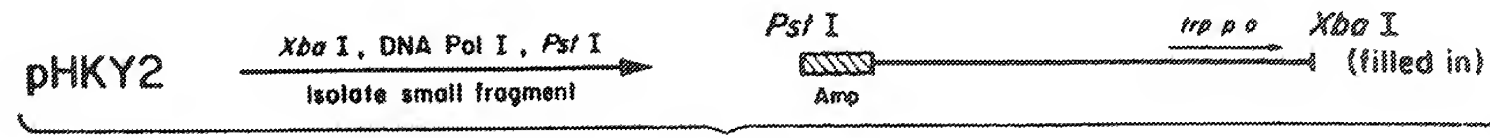

FIG.6(c)

MATURE HUMAN FIBROBLAST INTERFERON

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation of application Ser. No. 889,722, filed Jul. 28, 1986, now abandoned which is a divisional of application Ser. No. 291,892, now abandoned filed Aug. 11, 1981, which is a continuation-in-part of application Ser. No. 190,799, filed Sep. 25, 1980, now abandoned.

FIELD OF THE INVENTION

This invention relates to the microbial production, via recombinant DNA technology, of human fibroblast interferon for use in the treatment of viral and neoplastic diseases, and to the means and end products of such production.

BACKGROUND OF THE INVENTION

The publications and other materials referred to herein to illuminate the background of the invention and, in particular cases, to provide additional detail respecting its practice are incorporated herein by reference and, for convenience, are numerically referenced in the following text and respectively grouped in the appended bibliography.

Recombinant DNA Technology

With the advent of recombinant DNA technology, the controlled microbial production of an enormous variety of useful polypeptides has become possible. Already in hand are bacteria modified by this technology to permit the production of such polypeptide products such as somatostatin, the (components) A and B chains of human insulin, human growth hormone. More recently, recombinant DNA techniques have been used to occasion the bacterial production of proinsulin, thymosin alpha 1, (an immune potentiating substance produced by the thymus) and leukocyte interferon.

The workhorse of recombinant DNA technology is the plasmid, a non-chromosomal loop of double-stranded DNA found in bacteria and other microbes, oftentimes in multiple copies per cell. Included in the information encoded in the plasmid DNA is that required to reproduce the plasmid in daughter cells (i.e., a "replicon") and ordinarily, one or more selection characteristics such as, in the case of bacteria, resistance to antibiotics which permit clones of the host cell containing the plasmid of interest to be recognized and preferentially grown in selective media. The utility of plasmids lies in the fact that they can be specifically cleaved by one or another restriction endonuclease or "restriction enzyme", each of which recognizes a different site on the plasmidic DNA. Thereafter heterologous genes or gene fragments may be inserted into the plasmid by endwise joining at the cleavage site or at reconstructed ends adjacent to the cleavage site. DNA recombination is performed outside the cell, but the resulting "recombinant" plasmid can be introduced into it by a process known as transformation and large quantities of the heterologous gene-containing recombinant plasmid obtained by growing the transformant. Moreover, where the gene is properly inserted with reference to portions of the plasmid which govern the transcription and translation of the encoded DNA message, the resulting expression vehicle can be used to actually produce the polypeptide sequence for which the inserted gene codes, a process referred to as expression.

Expression is initiated in a region known as the promoter which is recognized by and bound by RNA polymerase. In some cases, as in the tryptophan or "trp" promoter preferred in the practice of the present invention, promoter regions are overlapped by "operator" regions to form a combined promoter-operator. Operators are DNA sequences which are recognized by so-called repressor proteins which serve to regulate the frequency of transcription initiation at a particular promoter. The polymerase travels along the DNA, transcribing the information contained in the coding strand from its 5' to 3' end into messenger RNA which is in turn translated into a polypeptide having the amino acid sequence for which the DNA codes. Each amino acid is encoded by a nucleotide triplet or "codon" within what may for present purposes be referred to as the "structural gene", i.e. that part which encodes the amino acid sequence of the expressed product. After binding to the promoter, the RNA polymerase first transcribes nucleotides encoding a ribosome binding site, then a translation initiation or "start" signal (ordinarily ATG, which in the resulting messenger RNA becomes AUG), then the nucleotide codons within the structural gene itself. So-called stop codons are transcribed at the end of the structural gene whereafter the polymerase may form an additional sequence of messenger RNA which, because of the presence of the stop signal, will remain untranslated by the ribosomes. Ribosomes bind to the binding site provided on the messenger RNA, in bacteria ordinarily as the mRNA is being formed, and themselves produce the encoded polypeptide, beginning at the translation start signal and ending at the previously mentioned stop signal. The desired product is produced if the sequences encoding the ribosome binding site are positioned properly with respect to the AUG initiator codon and if all remaining codons follow the initiator codon in phase. The resulting product may be obtained by lysing the host cell and recovering the product by appropriate purification from other bacterial protein.

Fibroblast Interferon

Human fibroblast interferon (FIF) is an antiviral protein which also exhibits a wide range of other biological activities (see ref. 1 for review). It has reportedly been purified to homogeneity as a single polypeptide of 19,000–20,000 molecular weight having a specific activity of 2 to 10×10^8 units/mg (2,3). The sequence of the 13 NH_2-terminal amino acids of FIF has been determined (4). Houghton et al. (5) have used synthetic deoxyoligonucleotides (predicted from this amino acid sequence) to determine the sequence of the 276 5'-terminal nucleotides of FIF mRNA. Taniguchi et al. (6) and Derynck et al. (7) have recently employed RNA selection procedures to identify cloned cDNA copies of FIF mRNA in *E. coli*. See also Taniguchi et al., *Gene* 10, 11 (1980) and *Proc. Natl. Acad. Sci. (U.S.A.)* 77, 5230 (1980) and *Nature* 285, 547 (1980).

While isolation from donor fibroblasts has provided sufficient material for partial characterization and limited clinical studies with homogeneous fibroblast interferon, it is a totally inadequate source for the amounts of interferon needed for large scale clinical trials and for broad scale prophylactic and/or therapeutic use thereafter. Indeed, presently clinical investigations employing human fibroblast-derived interferon in antitumor and antiviral testing have principally been confined to crude (<1 percent pure) preparations of the material, and long lead times for the manufacture of sufficient quantities, even at unrealistic price levels, have critically delayed investigation on an expanded front.

We perceived that application of recombinant DNA technology would be the most effective way of providing large

quantities of fibroblast interferon which, despite the absence in material so produced of the glycosylation characteristic of human-derived material, could be employed clinically in the treatment of a wide range of viral and neoplastic diseases.

More particularly, we proposed and have since succeeded in producing mature human fibroblast interferon microbially, by constructing a gene therefor which could then be inserted in microbial expression vehicles and expressed under the control of microbial gene regulatory controls.

Our approach to obtaining a fibroblast gene involved the following tasks:

1. Partial amino acid sequences would be obtained by characterization of fibroblast interferon purified to essential homogeneity, and sets of synthetic DNA probes constructed whose codons would, in the aggregate, represent all the possible combinations capable of encoding the partial amino acid sequences.
2. Bacterial colony banks would be prepared containing cDNA from induced messenger RNA. The probes of part (1) would be used to prime the synthesis of radio-labelled single stranded cDNA for use as hybridization probes. The synthetic probes would hybridize with induced mRNA as template and be extended by reverse transcription to form induced, radio-labelled cDNA. Clones from the colony bank that hybridized to radio-labelled cDNA obtained in this manner would be investigated further to confirm the presence of a full-length interferon encoding gene. Any partial length putative gene fragment obtained would itself be used as a probe for the full-length gene.
3. The full-length gene obtained above would be tailored, using synthetic DNA, to eliminate any leader sequence that might prevent microbial expression of the mature polypeptide and to permit appropriate positioning in an expression vehicle relative to start signals and the ribosome binding site of a microbial promoter. Expressed interferon would be purified to a point permitting confirmation of its character and determination of its activity notwithstanding the absence of glycosylation.

SUMMARY OF INVENTION

A series of replicable plasmidic expression vehicles have been constructed which direct the high level synthesis in transformant microorganisms of a mature polypeptide with the properties of authentic human fibroblast interferon. The product polypeptide exhibits the amino acid sequence of such interferon and is active in in vitro testing despite the lack of glycosylation characteristic of the human-derived material. Reference herein to the expression of "mature fibroblast interferon," connotes the bacterial or other microbial production of an interferon molecule unaccompanied by associated glycosylation and the presequence that immediately attends mRNA translation of the human fibroblast interferon genome. Mature fibroblast interferon, according to the present invention, is immediately expressed from a translation start signal (ATG) which also encodes the first amino acid codon of the natural product. The presence or absence of the methionine first amino acid in the microbially expressed product is governed by a kinetic phenomenon dependent on fermentation growth conditions and/or levels of expression in the transformant host. Mature fibroblast interferon could be expressed together with a conjugated protein other than the conventional leader, the conjugate being specifically cleavable in an intra- or extracellular environment. See British Patent Publication No. 2007676A. Finally, the mature interferon could be produced in conjunction with a microbial "signal" peptide which transports the conjugate to the cell wall, where the signal is processed away and the mature polypeptide secreted.

DESCRIPTION OF THE FIGURES

FIG. 1 depicts the protein sequence information used to design degenerate dodecanucleotide primers. Below the protein sequence are corresponding mRNA sequences and six pools of complementary deoxyoligonucleotide primers.

FIGS. 2A–C are autoradiographs of nitrocellulose-bound plasmid DNA prepared from some of the 600 bacterial transformants having DNA from the fibroblast cDNA library. The plasmid DNA of the nitrocellulose membrane was hybridized with either probe A, B, or C.

FIG. 3 presents DNA sequence of the cDNA insert of clone pFIF3 as determined by the Maxam-Gilbert procedure. The deduced protein sequence in printed above the DNA sequence.

FIG. 4 is a diagram showing the approach used to remove the signal peptide coding regions from pFIF3.

FIG. 5 is an autoradiograph of the polyacrylamide gel used to isolate the 141bp fragment lacking the signal peptide coding regions.

FIG. 6, and 6*a*, 6*b*, 6*c* schematically depicts the construction of plasmids coding for the direct expression of mature fibroblast interferon. Restriction sites and residues are as shown ("Pst I", etc.). "Ap^R" and "Tc^R" connote portions of the plasmid (s) which express, respectively, ampicillin and tetracycline resistance. The legend "p o" is an abbreviation for "promoter operator."

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Microorganisms Employed

The work described involved used of the microorganism: *E. coli* K-12 strain 294 (end A, thi$^-$, hsr$^-$, hsm$_k^+$), as described in British Patent Publication No. 2055382 A. This strain has been deposited on Oct. 28, 1978 with the American Type culture Collection, which is located at 12301 Parklawn Drive, Rockville, Md. 20852, and given ATCC Accession No. 31446. All recombinant DNA work was performed in compliance with applicable guidelines of the National Institutes of Health.

The invention, in its most preferred embodiments, is described with reference to *E. coli*, including not only strain *E. coli* K-12 strain 294, defined above, but also other known *E. coli* strains such as *E. coli* B, *E. coli x* 1776 and *E. coli* W 3110, or other microbial strains many of which are deposited and (potentially) available from recognized microorganism depository institutions, such as the American Type Culture Collection (ATCC)—*cf.* the ATCC catalogue listing. See also German Offenlegungschrift 2644432. These other microorganisms include, for example, Bacilli such as Bacillus subtilis and other enterobacteriaceae among which can be mentioned as examples Salmonella typhimurium and Serratia marcesans, utilizing plasmids that can replicate and express heterologous gene sequences therein. Yeast, such as Saccharomyces cerevisiae, may also be employed to advantage as host organism in the preparation of the interferon proteins hereof by expression of genes coding therefor under the control of a yeast promoter.

MATERIALS AND METHODS

General methods.

Restriction enzymes were purchased from New England Biolabs and used as directed. Plasmid DNA was prepared by a standard cleared lysate procedure (8) and purified by column chromatography on Biogel A-50M (Bio-Rad). DNA sequencing was performed using the method of Maxam and Gilbert (9). DNA restriction fragments were isolated from polyacrylamide gels by electroelution. DNA fragments were radiolabeled for use as hybridization probes by the random calf thymus DNA priming procedure of Taylor et al. (10). In situ colony hybridizations were performed by the Grunstein-Hogness procedure (11).

Chemical synthesis of deoxyoligonucleotides.

The deoxyoligonucleotides were synthesized by the modified phosphotriester method in solution (12), using trideoxynucleotides as building block (13). The material and general procedures were similar to those described (14). The six pools of primers (Fib 1–6) containing four dodecanucleotides each were obtained by separately coupling two hexamer pools (of two different 5'-terminal sequences each) with three different hexamer pools (of two different 3'-terminal sequences each).

Induction of fibroblasts.

Human fibroblasts (cell line GM-2504A) were grown as described previously (15). Growth medium (Eagles's minimal essential medium containing 10 percent fetal calf serum) was removed from roller bottles (Corning, 850 cm^2) and replaced with 50 ml growth medium containing 50 μg/ml of poly (I):poly (C) (PL Biochemicals) and 10 μg/ml cycloheximide. This induction medium was removed after 4 hours at 37° C. and cell monolayers were washed with PBS (0.14M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). Each bottle was incubated at 37° C. with 10 ml of a trypsin-EDTA solution (Gibco 610-5305) until cells were detached, and fetal calf serum was added to a concentration of 10 percent. Cells were spun for 15 minutes at 500× g and pellets were resuspended in PBS, pooled, and resedimented. Cells were frozen in liquid nitrogen. Approximately 0.17 g of cells were obtained per roller bottle.

Preparation and assay of interferon mRNA.

Poly extraction and oligo(dT)-cellulose chromatography as described elsewhere (16). The poly (A) containing RNA was enriched for interferon mRNA by centrifugation on a linear 5 percent to 20 percent (w/v) sucrose gradient. The RNA samples were heated to 80° C. for 2 minutes, rapidly cooled, layered over the gradient, and centrifuged for 20 hours at 30,000 rpm at 4° C. in a Beckman SW-40 rotor. Fractions were collected, ethanol precipitated, and dissolved in H_2O.

One microgram samples of mRNA were injected into *Xenopus laevis* oocytes as described previously (17,18). The injected oocytes were incubated 24 hours at 21° C., homogenized, and centrifuged for 5 minutes at 10,000× g. The interferon in the supernatant was determined by the cytopathic effect (CPE) inhibition assay (1) using Sindbis virus and human diploid (WISH) cells. Interferon titers of 1,000 to 6,000 units recovered (NIH reference standard) per microgram of RNA injected were routinely obtained for the 12S species of mRNA.

Synthesis and cloning of cDNA.

Single stranded cDNA was prepared in 100 μl reactions containing 5 μg of 12S fraction mRNA, 20 mM Tris-HCl (pH 8.3), 20 mM KCl, 8 mM $MgCl_2$, 30 mM β-mercaptoethanol, 100 μCi of (α^{32}p)dCTP (Amersham) and 1 mM dATP, dCTP, dGTP, dTTP. The primer was the synthetic Hind III decamer dCCAAGCTTGG (19), which had been extended at the 3' terminus with about 20 to 30 deoxythymidine residues using terminal deoxynucleotidyl transferase (20). 100 units of AMV reverse transcriptase were added and the reaction mixture was incubated at 42° C. for 30 minutes. The second strand DNA synthesis was carried out as described previously (21). The double stranded cDNA was treated with 1200 units of S1 nuclease (Miles Laboratories) for 2 hours at 37° C. in 25 mM sodium acetate (pH 4.5), 1 mM $ZnCl_2$, 0.3M NaCl. After phenol extraction the mixture was separated electrophoretically on an 8 percent polyacrylamide gel. cDNA (~0.5 μg) ranging from 500 to 1500 base pairs in size was recovered by electroelution. A 20 ng aliquot was extended with deoxyC residues using terminal deoxynucleotidyl transferase (20), and annealed with 100 ng of pBR322 which had been cleaved with Pst I and tailed with deoxyG residues (20). The annealed mixture was used to transform *E. coli* k-12 strain 294 (22) by a published procedure (23). Strain 294 was used throughout in the work described here, and has been deposited with the American Type Culture collection, accession no. 31446.

Preparation of induced and uninduced ^{32}p-cDNA probes.

5 μg of 12S mRNA were combined with either 2 μg of oligo $(dT)_{12-18}$ (Collaborative Research) or 5 μg of each synthetic primer pool (Fib 1 to Fib 6) in 60 μl of 10 mM Tris-HCl (ph 8), 1 mM EDTA. The mixtures were boiled 3 minutes, and quenched on ice. 60 μl of 40 mM Tris-HCl (pH 8.3), 40 mM KCl, 16 mM $MgCl_2$,60 mM β-mercaptoethanol, 1 mM dATP, dGTP, dTTP and 5×10^{-7}M (α-^{32}p) dCTP (Amersham, 2,000–3,000 Ci/mmole) was added to each template-primer mix at 0° C. After the addition of 100 units of AMV reverse transcriptase, the reactions were incubated at 42° C. for 30 minutes and purified by passage over 10 ml Sephadex G-50 columns. The products were treated with 0.3N NaOH for 30 minutes at 70° C., neutralized, and ethanol precipitated.

The ^{32}p-cDNAs were combined with 100 μg of poly (A) mRNA from uninduced fibroblasts in 50 μl of 0.4M sodium phosphate (pH6.8), 0.1 percent SDS. The mixtures were heated at 98° C. for 5 minutes and allowed to anneal 15 hours at 45° C. The DNA-RNA hybrids. (containing uninduced cDNA sequences) were separated from single-stranded DNA (induced cDNA sequences) by chromatography on hydroxyapatite as described by Galau et al. (24). The DNA-RNA hybrids were treated with alkali to remove RNA.

Screening of recombinant plasmids with ^{32}p-cDNA probes.

Approximately 1 μg samples of plasmid DNA were prepared from individual transformants by a published procedure (25). The DNA samples were linearized by digestion with Eco RI, denatured in alkali, and applied to each of three nitrocellulose filters (Schleicher and Schuell, BA85) by the dot hybridization procedure (26). The filters were hybridized with the ^{32}p-cDNA probes for 16 hours at 42° C. in 50 percent formamide, 10× Denhardt's solution (27), 6×SSC, 40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 40 μg/ml yeast RNA. Filters were washed with 0.1×SSC, 0.1 percent SDS twice for 30' at 42° C., dried, and exposed to Kodak XR-2 x-ray film using Dupont Lightning-Plus intensifying screens at −80° C.

Construction of plasmids for direct expression of FIF.

The synthetic primers I (dATGAGCTACAAC) and II (dCATGAGCTACAAC) were phosphorylated using T4 polynucleotide kinase and (γ^{32}.P)ATP (Amersham) to a specific activity of 700 Ci/mmole as described previously (28). Primer repair reactions were performed as follows: 250 pmoles of the ^{32}P-primers were combined with 8 μg (10 pmole) of a 1200 bp Hha I restriction fragment containing the FIF cDNA sequence. The mixture was ethanol precipitated, resuspended in 50 μl H_2O, boiled 3 minutes, quenched in a dry ice-ethanol bath, and combined with a 50 μl solution

of 20 mM Tris-HCl (pH 7.5), 14 mM $MgCl_2$, 120 mM NaCl, 0.5 mM dATP, dCTP, dGTP, dTTP at 0° C. 10 units of DNA polymerase I Klenow fragment (Boehringer-Mannheim) were added and the mixture was incubated at 37° C. for 4½ hours. Following extraction with phenol/$CHCl_3$ and restriction with Pst I, the desired product was purified on a 6 percent polyacrylamide gel. Subsequent ligations were done at room temperature (cohesive termini) or 4° C. (blunt ends) using previously detailed conditions (21,28).

Assay for interferon expression in *E. coli*.

Bacterial extracts were prepared for IF assay as follows: One ml cultures were grown overnight in LB (29) containing 5 μg/ml tetracycline, then diluted into 25 ml of M9 medium (29) containing 0.2 percent glucose, 0.5 percent casamino acids and 5 μg/ml tetracycline. 10 ml samples were harvested by centrifugation when A_{550} (Absorbance at 500 nanometers) reached 1.0. The cell pellets were quickly frozen in a dry ice-ethanol bath and cleared lysates were prepared as described by Clewell (8). Interferon activity in the supernatants was determined by comparison with NIH FIF standards using cytopathic effect (CPE) inhibition assays as reviewed previously (1). Two different assays were used: (a) WISH (human amnion) cells were seeded in microtiter dishes. Samples were added 16 to 20 hours later and diluted by serial 2-fold dilution. Sindbis virus was added after at least 3 hours in incubation. Plates were stained 20 to 24 hours later with crystal violet. (b) MDBK (bovine kidney) cell line was seeded simultaneously with 2-fold dilutions of samples. Vesicular stomatitis virus was added after 2 to 3 hours incubation and plates were stained with crystal violet 16 to 18 hours later. To test pH 2 stability bacterial extracts and standards were diluted in minimal essential medium to a concentration of 1000 units/ml. One ml aliquots were adjusted to pH 2 with 1N HCl, incubated at 4° C. for 16 hours, and neutralized by addition of NaOH. IF activity was determined by the CPE inhibition assay using human amnion cells. To establish antigenic identity 25 μl aliquots of the 1000 U/ml interferon samples (untreated) were incubated with 25 μl of rabbit antihuman leukocyte interferon for 60' at 37° C., centrifuged at 12,000× g for 5 minutes and the supernatant assayed. Fibroblast and leukocyte interferon standards were obtained from the National Institutes of Health. Rabbit antihuman leukocyte interferon was obtained from the National Institute of Allergy and Infectious Diseases.

RESULTS

Chemical synthesis of primer pools complementary of FIF mRNA.

The amino-terminal protein sequence of human fibroblast interferon (4) permitted us to deduce the 24 possible mRNA sequences which could code for the first four amino acids. The 24 complementary deoxyoligonucleoides were synthesized in 6 pools of 4 dodecamers each (FIG. 1).

The six pools of 4 deoxyoligonucleotides each were synthesized by a modified phosphotriester method that has been used previously for the rapid synthesis of oligonucleotides in solution (12) and on solid phase (14). The basic strategy involved reacting two different 3'-blocked trimers with an excess of a single 5'-protected trimer to yield a pool of two hexamers, each represented equally. The coupling of two pools, each containing two hexamers, then resulted in a pool of four dodecamers.

Identification of FIF cDNA clones.

Using 12S mRNA from induced human fibroblasts (1,000 units IF activity per μg in oocyte assay), double stranded cDNA was prepared and inserted into pBR322 at the Pst I site by the standard dG:dC tailing method (20). A fibroblast cDNA library consisting of 30,000 ampicillin-sensitive, tetracycline-resistant transformants of *E. coli* K-12 strain 294 was obtained from 20 ng of cDNA ranging in size from 550 to 1300 base pairs. Plasmid DNA was prepared from 600 of the transformants and applied to 3 sets of nitrocellulose filters as described in Materials and Methods.

The approach followed in the identification of hybrid plasmids containing fibroblast interferon cDNA sequences was similar to that used to identify human leukocyte interferon recombinant plasmids (30). Radiolabeled cDNA hybridization probes were prepared using either the 24 synthetic dodecamers or oligo$(dT)_{12-18}$ as primers and 12S RNA from induced fibroblasts (5000 units/μg in oocytes) as template. The ^{32}P-cDNAs (specific activity >5×10^8 cpm/μg) obtained were hybridized to a large excess of mRNA isolated from uninduced human fibroblasts, and the mRNA-cDNA hybrids were separated from unreacted cDNA by hydroxyapatite chromatography (24). The single stranded cDNA fractions should be enriched for sequences which are present in induced fibroblasts but absent in uninduced cells, and the mRNA-cDNA hybrids should represent sequences common to both induced and uninduced cells. Approximately 4×10^6 cpm of single stranded cDNA (hybridization probe A) and 8×10^6 cpm of cDNA-mRNA hybrids were obtained using oligo$(dT)_{12-18}$ primed cDNA; 1.5×10^6 cpm of single stranded (hybridization probe B) and 1.5×10^5 cpm of hybrids were obtained from cDNA primed using synthetic dodecamer pools Fib 1–6. The cDNA-mRNA hybrids from both fractionations were combined, the RNA hydrolyzed by treatment with alkali, and the ^{32}P-cDNA used as hybridization probe C. Many of the 600 plasmid samples hybridized with both probes A and C, indicating that the hybridization reactions between uninduced mRNA and ^{32}P-cDNA (prior to the hydroxyapatite fractionation step) had not gone to completion. However, only one of the 600 plasmids (pF526) hybridized strongly with the specifically primed, induced cDNA probe B (FIG. 2). Plasmid pF526 also hybridized with the total oligo$(dT)_{12-18}$ primed, induced cDNA probe A, and failed to give detectable hybridization to the combined uninduced probe C.

Pst I digestion of pF526 showed the cloned cDNA insert to be about 550 base pairs long, probably too short to contain the entire coding region for a protein the size of fibroblast interferon. Therefore, a ^{32}P-labeled DNA probe was prepared from this Pst I fragment by random priming with calf thymus DNA (10). This probe was used to screen 2000 individual colonies from a newly constructed fibroblast cDNA library (the new cDNA library was prepared using 12S mRNA from induced fibroblasts having a titer of 6,000 units/ml in the oocyte assay system). Sixteen clones hybridized to the probe. Plasmids prepared from the majority of these released two fragments when cleaved with Pst I, indicating that the cDNA contained an internal Pst I site. Clone pFIF3 contained the largest cDNA insert, about 800 base pairs. The DNA sequence of the insert was determined by the Maxam-Gilbert procedure (9) and is shown in FIG. 3. The amino acid sequence of human fibroblast interferon predicted from the nucleotide sequence is identical to that reported recently by Taniguchi et al. (31) and by Derynck et al. (7) from DNA sequencing of FIF cDNA clones. A precursor or signal peptide of 21 amino acids is followed by a mature interferon polypeptide of 166 amino acids, a stretch of 196 3'-untranslated nucleotides and a poly(A) tail. The NH_2-terminal 20 amino acids of mature FIF have been directly determined by protein microsequencing and are the same as those predicted from the DNA sequence. The calculated formula molecular weight of mature human fibro-

blast interferon having the 166 amino acids shown in FIG. 3 is about 20,027.

Direct expression of fibroblast interferon.

To express high levels of mature fibroblast interferon in *E. coli*, initiation of protein synthesis must occur at the ATG codon of the mature polypeptide (amino acid 1) rather than at the ATG of the signal peptide (amino acid S1) (FIG. 3).

Our approach to removing the signal peptide coding regions from pFIF3 is depicted in FIG. 4. A 1200 bp DNA fragment which contained the entire cDNA insert was isolated from a polyacrylamide gel after digesting pFIF3 with Hha I. Two separate synthetic deoxyoligonucleotide primers, dATGAGCTACAAC(I) and dCATGAGCTACAAC(II), were prepared. Both primers contain the coding sequence for the first four amino acids of mature fibroblast interferon; primer II has an additional C at the 5'-terminus. Primer repair reactions and subsequent ligations were carried out separately for primers I and II, and gave nearly identical results. Therefore, only reactions using primer I are discussed in detail here. The primers were 5'-radiolabeled using (γ-^{32}P)ATP and T4 polynucleotide kinase, combined with the 1200 bp Hha I DNA fragment and the mixture denatured by boiling. Following hybridization of the primer to the denatured Hha I DNA fragment, *E. coli* DNA polymerase I Klenow fragment (33) was used to catalyze the repair synthesis of the plus (top) strand (FIG. 4). In addition, the associated 3'≧5' exonuclease activity of the Klenow fragment removed the 3'-protruding end from the minus (bottom) strand, leaving a flush end. Analysis of samples of the reaction mixture by polyacrylamide gel electrophoresis indicated that the repair synthesis did not go to completion, but stopped at several discrete sites. Therefore, the entire reaction mixture was treated with Pst I and the desired 141 bp fragment (180,000 Cerenkov cpm; ~0.3 pmole) was purified by polyacrylamide gel electrophoresis (FIG. 5). Ligation of this fragment to 1 μg (~4 pmole) of the 363 bp Pst I-Bgl II fragment isolated from pFIF3 (FIG. 4), followed by Bgl II digestion, yielded 50,000 Cerenkov cpm (~0.1 pmole, ~30 ng) of the 504 bp DNA fragment containing the entire coding sequence for mature fibroblast interferon. The same reactions using primer II gave 83,000 cpm (~0.15 pmole, ~50 ng) of 505 bp product.

The construction of plasmids which direct the synthesis of human fibroblast interferon is outlined in FIG. 6. Separate expression plasmids were constructed which placed FIF synthesis under the control of the *E. coli* lac or trp promoter-operator systems. Both of these systems have proven useful for the direct expression of eukaryotic genes in *E. coli*: human growth hormone has been efficiently synthesized using the lac system (21) and human leukocyte interferon has been produced at high levels using the trp system (30) and *Nature* 287, 411 (1980).

pBRH trp was digested with EcoRI restriction enzyme and the resulting fragment isolated by PAGE and electroelution. EcoRI-digested plasmid pSom 11 (K. Itakura et al., *Science* 198, 1056 (1977); G.B. patent publication no. 2 007 676 A) was combined with the above fragment. The mixture was ligated with T_4 DNA ligase as previously described and the resulting DNA transformed into *E. coli* K-12 strain 294 as previously described. Transformant bacteria were selected on ampicillin-containing plates. Resulting ampicillin-resistant colonies were screened by colony hybridization (M. Grunstein et al., Proc Nat'l Acad Sci USA 72, 3951–3965 (1975)) using as a probe the trp promoter-operator-containing the above fragment isolated from pBRHtrp, which had been radioactively labelled with p^{32}. Several colonies shown positive by colony hybridization were selected, plasmid DNA was isolated and the orientation of the inserted fragments determined by restriction analysis employing restriction enzymes BglII and BamHI in double digestion. *E. coli* 294 containing the plasmid designated pSOM7Δ2, which has the trp promoter-operator fragment in the desired orientation was grown in LB medium containing 10 μg/ml ampicillin. The cells were grown to optical density 1 (at 550 nM), collected by centrifugation and resuspended in M9 media in tenfold dilution. Cells were grown for 2–3 hours, again to optical density 1, then lysed and total cellular protein analyzed by SDS (sodium dodecyl sulfate) urea (15 percent) polyacrylamide gel electrophoresis (J. V. Maizel Jr. et al., Meth Viral 5, 180–246 (1971)).

Plasmid pBR322 was Hind III digested and the protruding Hind III ends in turn digested with S1 nuclease. The S1 nuclease digestion involved treament of 10 μg of Hind III-cleaved pBR322 in 30 μl S1 buffer (0.3M NaCl, 1 mM $ZnCl_2$, 25 mM sodium acetate, pH 4.5) with 300 units S1 nuclease for 30 minutes at 15° C. The reaction was stopped by the addition of 1 μl of 30×S1 nuclease stop solution (0.8M tris base, 50 mM EDTA). The mixture was phenol extracted, chloroform extracted and ethanol precipitated, then EcoRI digested as previously described and the large fragment (1) obtained by PAGE procedure followed by electroelution. The fragment obtained has a first EcoRI sticky end and a second, blunt end whose coding strand begins with the nucleotide thymidine.

Plasmid pSom7 Δ2, as prepared above, was Bgl II digested and the Bgl II sticky ends resulting made double stranded with the Klenow polymerase I procedure using all four deoxynucleotide triphosphates. EcoRI cleavage of the resulting product followed by PAGE and electroelution of the small fragment (2) yielded a linear piece of DNA containing the tryptophan promoter-operator and codons of the LE' "Proximal" sequence upstream from the Bgl II site ("LE'(p)"). The product had an EcoRI end and a blunt end resulting from filling in the Bgl II site. However, the Bgl II site is reconstituted by ligation of the blunt end of the above fragment (2) to the blunt end of the above prepared fragment (1). Thus, the two fragments were ligated in the presence of T_4 DNA ligase to form the recirculated plasmid pHKY 10 which was propagated by transformation into competent *E. coli* strain 294 cells.

Plasmid pGM1 carries the *E. coli* tryptophan operon containing the deletion ΔLE1413 (G. F. Miozzari, et al., (1978) *J. Bacteriology* 133, 1457–1466)) and hence expresses a fusion protein comprising the first 6 amino acids of the trp leader and approximately the last third of the trp E polypeptide (hereinafter referred to in conjunction as LE'), as well as the trp D polypeptide in its entirety, all under the control of the trp promoter-operator system. The plasmid, 20 μg, was digested with the restriction enzyme PvuII which cleaves the plasmid at five sites. The gene fragments were next combined with EcoRI linkers (consisting of a self complementary oligonucleotide of the sequence: pCATGAATTCATG) providing an EcoRI cleavage site for a later cloning into a plasmid containing an EcoRI site. The 20 μg of DNA fragments obtained from pGM1 were treated with 10 units T_4 DNA ligase in the presence of 200 pico moles of the 5'-phosphorylated synthetic oligonucleotide pCATGAATCATG and in 20 μl T_4 DNA ligase buffer (20 mM tris, pH 7.6, 0.5 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol) at 4° C. overnight. The solution was then heated 10 minutes at 70° C. to halt ligation. The linkers were cleaved by EcoRI digestion and the fragments, now with EcoRI ends were separated using 5 percent polyacrylamide gel electrophoresis (hereinafter "PAGE") and the three largest fragments

isolated from the gel by first sta... ...ng with ethidium bromide, locating the fragments with ultraviolet light, and cutting from the gel the portions of interest. Each gel fragment, with 300 microliters 0.1×TBE, was placed in a dialysis bag and subjected to electrophoresis at 100 v for one hour in 0.1× TBE buffer (TBE buffer comains: 10.8 gm tris base, 5.5 gm boric acid, 0.09 gm Na_2EDTA in 1 liter H_2O). The aqueous solution was collected from the dialysis bag, phenol extracted, chloroform extracted and made 0.2M sodium chloride, and the DNA recovered in water after ethanol precipitation. The trp promoter-operator-containing gene with EcoRI sticky ends was identified in the procedure next described, which entails the insertion of fragments into a tetracycline sensitive plasmid which, upon promoter-operator insertion, becomes tetracycline resistant.

Plasmid pBRH1 (R. J. Rodriguez, et al, Nucleic Acids Research 6, 3267–3287 [1979]) expresses ampicilin resistance and contains the gene for tetracycline resistance but, there being no associated promoter, does not express that resistance. The plasmid is accordingly tetracycline sensitive. By introducing a promoter-operator system in the EcoRI site, the plasmid can be made tetracycline resistant.

pBRH1 was digested with EcoRI and the enzyme removed by phenol extraction followed by chloroform extraction and recovered in water after ethanol precipitation. The resulting DNA molecule was, in separate reaction mixtures, combined with each of the three DNA fragments obtained above and ligated with T_4 DNA ligase as previously described. The DNA present in the reaction mixture was used to transform competent *E. coli* K-12 strain 294, K. Backman et al., Proc Nat'l Acad Sci USA 73, 4174–4198 [1976]) by standard techniques (V. Hershfield et al., Proc Nat'l Acad Sci USA 71, 3455–3459 [1974]) and the bacteria plated on LB plates containing 20 μg/ml ampicillin and 5 μg/ml tetracycline. Several tetracycline-resistant colonies were selected, plasmid DNA isolated and the presence of the desired fragment confirmed by restriction enzyme analysis. The resulting plasmid is designated pBRHtrp.

An EcoRI and BamHI digestion product of the viral genome of hepatitis B was obtained by conventional means and cloned into the EcoRI and BamHI sites of plasmid pGH6 (D. V. Goeddel et al., Nature 281, 544 [1979])) to form the plasmid pHS32. Plasmid pHS32 was cleaved with XbaI, phenol extracted, chloroform extracted and ethanol precipitated. It was then treated with 1 μl *E. coli* polymerase I, Klenow fragment (Boehringer-Mannheim) in 30 μl polymerase buffer (50 mM potassium phosphate pH 7.4, 7 mM $MgCl_2$, 1 mM β-mercaptoethanol) containing 0.1 mM dTTP and 0.1 mM dCTP for 30 minutes at 0° C. then 2 hr. at 37° C. This treatment causes 2 of the 4 nucleotides complementary to the 5' protruding end of the XbaI cleavage site to be filled in: t.0170

Two nucleotides, dC and dT, were incorporated giving an end with two 5' protruding nucleotides. This linear residue of plasmid pHS32 (after phenol and chloroform extraction and recovery in water after ethanol precipitation) was cleaved with EcoRI. The large plasmid fragment was separated from the smaller EcoRI-XbaI fragment by PAGE and isolated after electroelution. This DNA fragment from pHS32 (0.2 μg), was ligated, under conditions similar to those described above, to the EcoRI-Taq I fragment of the tryptophan operon (~0.0) μg), derived from pBRHtrp.

In the process of ligating the fragment from pHS32 to the Eco RI-Taq I fragment, as described above, the Taq I protruding end is ligated to the XbaI remaining protruding end even though it is not completely Watson-Crick base-paired: t.0180

A portion of this ligation reaction mixture was transformed into *E. coli* 294 cells, heat treated and plated on LB plates containing ampicillin. Twenty-four colonies were selected, grown in 3 ml LB media, and plasmid isolated. Six of these were found to have XbaI site regenerated via E. coli catalyzed DNA repair and replication: t.0181

These plasmids were also found to cleave both with EcoRI and HpaI and to give the expected restriction fragments. One plasmid, designated pTrp 14, was used for expression of heterologous polypeptides, as next discussed.

The plasmid pHGH 107 (D. V. Goeddel et al, *Nature*, 281, 544, 1979) contains a gene for human growth hormone made up of 23 amino acid codons produced from synthetic DNA fragments and 163 amino acid codons obtained from complementary DNA produced via reverse transcription of human growth hormone messenger RNA. This gene, though it lacks the codons of the "pre" sequence of human growth hormone, does contain an ATG translation initiation codon. The gene was isolated from 10 μg pHGH 107 after treatment with EcoRI followed by *E. coli* polymerase I Klenow fragment and dTTP and dATP as described above. Following phenol and chloroform extraction and ethanol precipitation the plasmid was treated with BamHI.

The human growth hormone ("HGH") gene-containing fragment was isolated by PAGE followed by electroelution. The resulting DNA fragment also contains the first 350 nucleotides of the tetracycline resistance structural gene, but lacks the tetracycline promoter-operator system so that, when subsequently cloned into an expression plasmid, plasmids containing the insert can be located by the restoration of tetracycline resistance. Because the EcoRI end of the fragment has been filled in by the Klenow polymerase I procedure, the fragment has one blunt and one sticky end, ensuring proper orientation when later inserted into an expression plasmid.

The expression plasmid pTrp14 was next prepared to receive the HGH gene-containing fragment prepared above. Thus, pTrp14 was XbaI digest and the resulting sticky ends filled in with the Klenow polymerase I procedure employing dATP, dTTP, dGTP and dCTP. After phenol and chloroform extraction and ethanol precipitation the resulting DNA was treated with BamHI and the resulting large plasmid fragment isolated by PAGE and electroelution. The pTrp14-derived fragment had one blunt and one sticky end, permitting recombination in proper orientation with the HGH gene containing fragment previously described.

The HGH gene fragment and the pTrp14 ΔXba-BamHI fragment were combined and ligated together under conditions similar to those described above. The filled in XbaI and EcoRI ends ligated together by blunt end ligation to recreate both the XbaI and the EcoRI site: t.0190

This construction also recreates the tetracycline resistance gene. Since the plasmid pHGH 107 expresses tetracycline resistance from a promoter lying upstream from the HGH gene (the lac promoter), this construction, designated pHGH 207, permits expression of the gene for tetracycline resistance under the control of the tryptophan promoter-operator. Thus the ligation mixture was transformed into *E. coli* 294 and colonies selected on LB plates containing 5 μg/ml tetracycline.

Plasmid pHGH 207 was EcoRI digested and the trp promoter containing EcoRI fragment recovered by PAGE followed by electroelution. Plasmid pBRH1 was EcoRI digested and the cleaved ends treated with bacterial alkaline phosphatase ("BAP") (1 μg, in 50 mM tris pH 8 and 10 mM $MgCl_2$ for 30 min. at 65° C.) to remove the phosphate groups on the protruding EcoRI ends. Excess bacterial

alkaline phosphatase was removed by phenol extraction, chloroform extraction and ethanol precipitation. The resulting linear DNA, because it lacks phosphates on the protruding ends thereof, will in ligation accept only inserts whose complementary stick ends are phosphorylated but will not itself recircularize, permitting more facile screening for plasmids containing the inserts.

The EcoRI fragment derived from pHGH 207 and the linear DNA obtained from pBRH1 were combined in the presence of T_4 ligase as previously described and ligated. A portion of the resulting mixture was transformed into *E. coli* strain 294 as previously described, plated on LB media containing 5 μg/ml of tetracycline, and 12 tetracycline resistant colonies selected. Plasmid was isolated from each colony and examined for the presence of a DNA insert by restriction endonuclease analysis employing EcoRI and XbaI. One plasmid containing the insert was designated pHKY1.

The plasmid pHKY10, described above, is a derivative of pBR322 which contains a Bgl II site between the tetracycline resistance (Tc^R) promoter and structural gene (32). The large DNA fragment isolated after digesting pHKY10 with Pst I and Bgl II therefore contains part of the ampicillin resistance (Ap^R) gene and all of the Tc^R structural gene, but lacks the Tc^R promoter (FIG. 6). The plasmid pGH6 (21) was digested with Eco RI, the resulting single stranded ends were filled in with DNA polymerase I, and the plasmid was cleaved with Pst I. The small fragment, containing part of the Ap^R gene, a double lac promoter and lac ribosome binding site, but lacking an ATG initiation triplet was isolated. A similar trp promoter fragment, containing the trp leader ribosome binding site, but lacking an ATG sequence (30), may be isolated from pHKY1 described above; see (32) (see FIG. 6).

The trp fragment just referred to is an analog of the *E. coli* tryptophan operon from which the so-called trp attenuator has been deleted. See *J. Bact.* 133, 1457 (1978), to controllably heighten expression levels. Expression plasmids containing the modified trp regulon can be grown to predetermined levels in nutrient media containing additive tryptophan in quantities sufficient to repress the promoter-operator system, then be deprived of tryptophan so as to derepress the system and occasion the expression of the intended product.

The expression plasmids may be assembled via three part ligation reactions as shown in FIG. 6. 15 ng (~0.05 pmole) of the assembled FIF gene (504 or 505 bp), 0.5 μg (~0.2 pmole) of the large Pst I-Bgl II fragment of pHKY10 and 0.2 μg (~0.3 pmole) of the appropriate promoter fragment were ligated and the mixture used to transform *E. coli* 294 (22). Plasmid DNA was prepared from individual transformants and analyzed by restriction mapping. Correct joining of the assembled gene to the promoter fragment should restore the Eco RI (lac) or the Xba I (trp) recognition sequences. The majority of the plasmids gave the expected restriction enzyme digestion patterns. Individual clones (12 containing the trp promoter and 12 containing the lac promoter) were grown and extracts prepared for interferon assay as described in Materials and Methods.

When assayed on human amnion (WISH) cells for antiviral activity by the CPE inhibition assay (1) five of the trp transformants were positive (each approximately equivalent); eleven of the lac transformants gave equivalent IF activities. Therefore, one transformant from each series (pFIFlac9 and pFIFtrp69) was selected for further study (Table 1). DNA sequence analysis demonstrated that the desired attachment of promoter to FIF structural gene had occurred in both cases. t.0216

The amounts of fibroblast interferon produced by pFIFlac9 and pFIFtrp69 are shown in Table 1. The trp promoter gave a FIF expression level measurably higher than did the lac promoter. In an attempt to further increase FIF expression levels, pFIFtrp69 was cleaved with Eco RI and two 300 base pair Eco RI fragments containing the trp promoter (30) were inserted. The resulting plasmid, pFIFtrp^{3}69, contains three successive trp promoters which read toward the FIF gene. The amount of FIF synthesized by *E. coli* K-12 strain 294/pFIF trp^{3}69 is 4–5 times that produced by pFIF trp 69 (Table 1). This is apparently due to the derepression of the trp promoter which occurs when trp repressor levels are titrated by the multiple copies of the trp operator.

The FIF produced by *E. coli* K-12 strain 294/pFIFtrp69 behaves like authentic human FIF. As shown in Table 2, its antiviral activity is about 30 times greater on human cells than bovine cells. In addition, the bacterially produced FIF is stable to treatment at pH 2 overnight and is not neutralized by rabbit antihuman leukocyte interferon antibodies (Table 3). t.0220 t.0221

Purification

The purification procedure for bacterial derived fibroblast is as follows:

1. Frozen cells are suspended in twelve times volume per weight with sucrose lysis buffer (100 mM Tris-HCl, 10 percent sucrose, 0.2M NaCl, 50 mM EDTA, 0.2 mM PMSF, pH 7.9) containing lysozyme at 1 mg ml^{-1}. The cell suspension is stirred for 1 hour at 4° C. and centrifuged. Fibroblast interferon activity remains in the supernatant.
2. Polyethyleneimine (5 percent v/v) is added to the sonicated supernatant to a final concentration of 0.5 percent (v/v). The solution is stirred for 1 hour at 4° C. and centrifuged. Interferon activity remains in the supernatant.
3. Solid ammonium sulfate is added to the polyethyleneimine supernatant to a final concentration of 50 percent saturation, stirred for 30 minutes at 4° C. and centrifuged. Interferon activity is in the 50 percent pellet.
4. The 50 percent ammonium sulfate pellet is suspended in one half the volume of the 50 percent ammonium sulfate suspension with Phosphate Buffered Saline (20 mM sodium phosphate 0.15M NaCl, pH 7.4). Polyethylene glycol 6000 (50 percent w/v in PBS) is added to a final concentration of 12½ percent (v/v), stirred at 4° C. for 2 hours and centrifuged. Interferon activity is in the pellet. The pellet is suspended in a minimal volume of sucrose lysis buffer and clarified by centrifugation.

This initial extraction procedure results in a purification of fibroblast interferon from 0.001 percent of the total protein to 0.05 percent of the total protein. This material can be further purified to homogeneity by the following column chromatography steps:

5. Affinity chromatography on Amicon Blue B in sucrose lysis buffer.
6. Anion exchange chromatography on QAE Sephadex in sucrose lysis buffer in the absence of 0.2M NaCl.
7. Size exclusion chromatography on Sephadex G-75 in sucrose lysis buffer.
8. Reverse phase high pressure liquid chromatography.

Parenteral Administration

FIF may be parenterally administered to subjects requiring antitumor or antiviral treatment. Dosage and dose rate may parallel that currently in use in clinical investigations of human derived materials, e.g., about $(1–10)\times10^6$ units daily, and in the case of materials of purity greater than 1 percent-

age, likely up to, e.g., 150×10^6 units daily. Dosages of bacterially obtained FIF could be significantly elevated for greater effect owning to the essential absence of human proteins other than FIF, which proteins in fibroblast-derived materials may act as pyrogens, exhibiting adverse effects, e.g., malaise, temperature elevation, etc.

As one example of an appropriate dosage form for essentially homogeneous bacterial FIF in parenteral form, 3 mg. FIF of specific activity of, say, 2×10^8 μ/mg may be dissolved in 25 ml. 5 percentage serum albumin (human) - USP, the solution passed through a bacteriological filter and the filtered solution aspetically subdivided into 100 vials, each containing 6×10^6 units pure interferon suitable for parenteral administration. The vials are preferably stored in the cold (−20° C.) prior to use.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the polypeptide hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described in Remington's *Pharmaceutical Sciences* by E. W. Martin, which is hereby incorporated by reference. Such compositions will contain an effective amount of the interferon protein hereof together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective administration to the host. One preferred mode of administration is parenteral.

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We claim:

1. A composition comprising water and a nonglycosylated polypeptide having the amino acid sequence of a mature human fibroblast interferon, said nonglycosylated polypeptide having a total of 165 or 166 amino acids and said composition being free of any glycosylated human fibroblast interferon.

2. The composition of claim 1, said nonglycosylated polypeptide having the amino acid sequence

X-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-Gln-Cys-Gln-Lys-Leu-Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-Pro-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-Ser-Thr-Gly-Trp-Asn-Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-Val-Leu-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-Lys-Glu-Tyr-Ser-His-Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-Thr-Gly-Tyr-Leu-Arg-Asn,

wherein X is H or Met.

3. The composition of claim 2, said nonglycosylated polypeptide having a formula molecular weight of about 20,027.

4. The composition of claim 1, 2 or 3, said composition being free of human proteins.

5. The composition of claim 1, 2 or 3, said composition containing a therapeutically effective amount of said nonglycosylated polypeptide and being suitable for parenteral administration.

6. The composition of claim 4, said composition containing a therapeutically effective amount of said nonglycosylated polypeptide and being suitable for parenteral administration.

* * * * *